STUDIES ON RECOMBINATION WITH INFLUENZA VIRUSES IN THE CHICK EMBRYO

I. INVASION OF THE CHICK EMBRYO BY INFLUENZA VIRUSES

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Three strains of influenza virus A have been described which have the capacity to invade the chick embryo after chorioallantoic inoculation and to produce death characteristically with intense "haemorrhagic encephalitis." These are the Melbourne and WS strains adapted to growth on the chorioallantois in this laboratory (MEL-Egg and WSE) (Burnet, 1936; Burnet and Bull, 1944; Burnet and Lush, 1938) and Stuart-Harris' strain neuro-flu or NWS (Stuart-Harris, 1939) also derived from WS.

Much work has recently been concerned with the use of NWS as one component in virus recombination experiments (Burnet and Lind, 1951; Burnet and Edney, 1951) and it was felt desirable that efforts to produce recombination in the allantoic cavity as well as in the mouse brain, should be made. The obvious approach would be to use the capacity of the NWS strain to invade the embryo as the equivalent of its neurotropism in mice. It was hoped that, by placing a mixture of NWS and a non-invasive strain such as MEL in the allantoic cavity, protecting the embryo meanwhile against invasion by the NWS with appropriate antiserum, and by isolating virus from the embryo brain, it should be possible to obtain an invasive MEL strain that might also be pathogenic for mice by intracerebral inoculation. Preliminary experiments, however, indicated that when NWS is grown in the allantoic cavity it very rarely invades the embryo and that when it does invade and kill the embryo there is frequently no detectable haemagglutinin in the allantoic fluid. This made it impossible to carry out the experiments as they were at first planned, but did raise a series of interesting problems.

A survey of the literature showed that there has been no serious discussion of the extent and nature of the invasion of the chick embryo by influenza virus after inoculation in the allantoic cavity. There are also other aspects of the distribution of virus in the chick embryo which are not easy to understand. Chorioallantoic inoculation, even with a virus which invades the embryo rarely produces detectable haemagglutinin in the allantoic cavity or in the

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amniotic sac. A properly performed intravenous injection with a small dose of virus also fails to show evidence of virus multiplication in the allantoic cavity when a lethal infection of the embryo is produced.

The immediate problem was to understand why NWS infections on the chorioallantois regularly produce fatal haemorrhagic lesions in the chick embryo while allantoic infections in general do not. In addition the possibility of a converse effect was raised by the finding, less frequently, of instances in which NWS inoculated into the allantoic cavity produced haemorrhagic brain lesions, but did not give rise to the appearance of measurable agglutinin in the allantoic fluid. It seemed likely that phenomena of this sort were common to any influenza virus strains of similar character for, in 1944, Burnet and Bull found very similar behaviour on the part of MEL-Egg which grew poorly in the allantoic cavity and often produced fatal infection of the embryo without haemagglutinin being produced in the allantoic fluid. With an ID_{50} of $10^{5 \cdot 5}$, when estimated by inoculation into the allantois, intravenous inoculation gave a value of $10^{7 \cdot 5}$, 100 times as high. Antiserum was much more successful in preventing the appearance of haemagglutinin in the allantoic cavity than in protecting against invasion of the embryo.

The experiments reported in the present paper, in addition to providing a background to the work on recombination discussed in the second and third papers, indicate that an established infection in the allantoic cavity will protect the embryo against the characteristic haemorrhagic lesions when NWS is given intravenously.

Materials and Methods.

Virus strains. The influenza A strains MEL (Burnet, 1935) and neuro-WS (NWS) of Stuart–Harris (1939) were reisolated from limiting dilutions and for each strain a pool of infected allantoic fluid was distributed in small ampoules which were stored in a dry ice cabinet. These stocks were used for all the experiments cited in the present paper.

Infectivity titrations. Inoculations by allantoic, chorioallantoic and intravenous routes were made according to standard methods described by Beveridge and Burnet (1946), all dilutions being made in ice-cold horse serum saline (10 p.c. horse serum). The only departure from the standard method of estimating infectivity titres was to include, for reasons which are apparent in the text, the incidence of death of the embryo or of characteristic embryonic lesions, as well as the incidence of "positive" allantoic fluids. Groups of four 11-day eggs were used which were examined for haemagglutinin at the 2nd, 3rd and 4th days and for specific deaths and lesions at the 4th day. Care was taken to prevent cross-infection of the eggs when testing for agglutinin. In practice it was found that fluids frequently became positive between the 2nd and 3rd days, but rarely between the 3rd and 4th days. End points are calculated by the Reed-Muench procedure.

Immune ferret sera. These were prepared from ferrets infected 10 days previously with active virus (MEL or NWS) given intranasally. The sera were inactivated at 56° C. and distributed in 1 ml. amounts which were stored frozen at —15° C. At the concentrations used the sera had insignificant action against the heterologous viruses.

Recovery of virus from embryo brains. In estimating the virus content of embryo brains account had to be taken of the fact that surrounding fluids often contained virus. Accordingly

the procedure adopted for the recovery of the brain specimen was to remove the chick embryo, flush the head in a stream of sterile saline and sear the cranium with a hot iron. Through this area from which infected skin was always removed and which frequently penetrated to the brain's surface the cranial contents were removed as completely as possible into a wide bore sterile Pasteur pipette. The contents were rubbed up with a small quantity of sterile alundum, emulsified in 2 ml. of sterile saline and, if not used immediately, placed in ampoules on dry ice until required. From this emulsion suitable dilutions were made for suballantoic inoculation and the expression "titre of virus in the brain" implies the titre of such an emulsion.

EXPERIMENTAL.

The behaviour of stock NWS strain.

The haemagglutinin titre of the pool was 180 with 1 p.c. fowl cells. Titration of the stock by the standard allantoic method gave the results in column one of Table 1, the ID_{50}, based on positive haemagglutination in the fluids being $10^{6.4}$. An analysis of those titrations in which eggs were examined at four days after inoculation both for embryonic lesions and for positive haemagglutination in the allantoic fluid, is shown in the second portion of Table 1. Of 25 eggs showing evidence of infection, 20 showed a positive fluid with living normal embryo, 4 a negative fluid with embryo dead with typical haemorrhagic lesions and only one showing both positive fluid and specifically dead embryo. Three of the negative fluids from dead embryos were titrated for infectivity; two gave titres of $10^{-3.0}$ and $10^{-2.0}$ respectively, the other produced one infection in four eggs inoculated with undiluted fluid.

TABLE 1.

Allantoic titration of stock NWS fluid.

Dilution	Positive Fluids	Analysis of detailed titration			
		F+E+	F+E—	F—E+	F—E—
10^{-8}	1/18	0	1	0	7
10^{-7}	9/19	0	3	1	3
10^{-6}	12/19	0	3	0	4
10^{-5}	13/20	1	3	0	2
10^{-4}	16/19	0	10	3	1
		(1)	(20)	(4)	

F+ = haemagglutinin present in allantoic fluid.
F— = haemagglutinin absent from allantoic fluid.
E+ = specific lesions present in embryo.
E— = specific lesions absent from embryo.

It is of incidental interest that, when neutralization tests with ferret immune serum are carried out in the allantoic cavity, an increase in the proportion of embryos with specific lesions but negative fluids is obtained. Control titration in horse serum saline gave an ID_{50} of $10^{-6.5}$, all the positive and

negative fluids being associated with normal embryos. The same virus diluted in immune serum F373 (anti-NWS) 1:50, gave an end point of $10^{-2.2}$ based on any evidence of infection. This included three embryos with haemorrhagic lesions but negative fluids and two with both lesions in the embryo and haemagglutinin in the fluid. These results recall earlier findings with MEL-Egg, referred to in the introduction to this paper.

When inoculated on to the chorioallantois, NWS produces moderately large pocks to a dilution of 10^{-6} and kills all infected embryos with haemmorhagic lesions in the brain and muscles, in 2 to 4 days, the time of survival depending on the dose of the inoculum. Intravenous inoculation gives the clearest end-point of any method of titration. Death of the embryo is produced in from two to three days with gross haemorrhagic encephalitis. A titration of the stock fluid by this method gave, with 0·1 ml. volumes of inoculum, at 10^{-6} 9/9, 10^{-7} 6/9, 10^{-8} 3/9, specific deaths, the ID_{50} being $10^{-7.5}$.

In summary, it will be seen that the embryo tissues, presumably the vascular endothelium in particular, are highly susceptible to infection by NWS. Free multiplication takes place in the embryo—an emulsion of embryo brain from a specimen with typical lesions shows an average titre of 10^{-5} to 10^{-6}. Further, it is clear that small amounts of virus readily reach the circulation from chorioallantoic lesions and from some at least of allantoic cavity infections.

The characteristics of stock MEL strain.

The haemagglutinating titre of the stock MEL virus used was 800 against 1 p.c. fowl cells, the infectivity titre $10^{-9.0}$ by the allantoic route. Most embryos inoculated with low dilutions of virus, 10^{0} to 10^{-2}, suballantoically die at the third or fourth day after inoculation without visible macroscopic lesions.

On the chorioallantois large concentrations of virus produce an oedematous and granular membrane and at high dilutions some membranes show tiny pocks. The embryos inoculated with higher dilutions appear normal at three and four days.

The results of intravenous inoculation have been variable, possibly depending on minor seasonal variations in the resistance of the embryos. With the largest practicable (non-toxic) dose, 0·1 ml. of 10^{-2} dilution of stock fluid all embryos inoculated died by the third day, 9/11 showing moderate to intense brain haemorrhage, the other two having no obvious lesions. With virus diluted 10^{-4} one experiment gave 3/4 embryos dying with specific haemorrhages at the 3rd to 4th days, the other surviving; another experiment gave 2/8 deaths without haemorrhage, one embryo alive with cutaneous haemorrhages and five alive without haemorrhages, but developmentally retarded. Seven embryos inoculated with virus at 10^{-6} gave one death with minor haemorrhagic lesions, one death without lesions and five living normal embryos. With small doses it is

usually possible to avoid infection of the allantoic fluid, but with a dilution of 10^{-2} all allantoic fluids develop haemagglutinin. A summary of the results is presented in Table 2.

TABLE 2.

The effect of intravenous inoculation of MEL virus into 12-day chick embryos.

Virus dilution	Number of embryos	Number of positive fluids (HA)	Embryo lesions		
			Spec. lesions	Non-spec. deaths	No lesion
10^{-6}	7	3	1	1	5
10^{-4}	8	4	1	2	5
10^{-4}	4	3	3	0	1
10^{-2}	11	11	9	2	0

The occurrence of gross brain haemorrhage after intravenous inoculation of MEL was unexpected and the possibility that a mutant form was responsible had to be excluded. Haemorrhagic brains were emulsified in saline and centrifuged. All the supernatant fluids showed small amounts of haemagglutinin inactivated by dilute anti-MEL ferret serum and were infective at a dilution beyond 10^{-4}. The *in vitro* characters of the fluids obtained from these titrations were identical with normal MEL. Four such brain emulsions diluted to 1/100 were inoculated intravenously into 12-day embryos which were opened and examined after three days' incubation. No lesions were evident, indicating that there had been no selection of a haemorrhage-producing mutant of MEL.

Moderate amounts of virus (10^{-1} to 10^{-4}) were also present in the brains of embryos showing no lesions after intravenous inoculation of small doses of MEL.

The passage of virus into the circulation after infection had been initiated by the allantoic or chorioallantoic route, was estimated by similar titrations of brain emulsions taken at appropriate times after inoculation. Table 3 shows that virus is almost always present, sometimes in considerable amount, from the second day onwards.

TABLE 3.

Virus MEL in the brain of embryos after allantoic or chorioallantoic inoculation.

Route of inoculation	Day	Result of inoculation of brain emulsions		
Allantoic	1	0/4	0/4	1/4
	2	4/4	4/4	4/4
	3	0/4	4/4(10^{-4})	—
	4	4/4(10^{-4})	4/4(10^{-3})	—
Chorioallantoic	1	0/4	1/4	3/4
	2	1/4	4/4	4/4
	3	4/4(10^{-1})	3/4	4/4(10^{-2})
	4	4/4(10^{-2})	4/4(10^{-4})	0/4

Figures indicate positive fluids/eggs inoculated, each column one brain.
Figures in brackets indicate approximate end-point when titration made.

To summarize, MEL virus, which can be regarded as a typical influenza A strain well adapted to allantoic passage, shows regular entry into the circulation. Small amounts reaching the brain produce no lesions, but a large intravenous inoculum produces the full picture of "haemorrhagic encephalitis." No neurotropic variant arises in this process. By whatever route inoculated, multiplying virus will eventually kill the embryo, but without causing macroscopic lesions.

Protection of the embryo by the presence of established infection in the allantoic cavity.

The failure of NWS infection in the allantoic cavity to induce haemorrhagic lesions in the embryo may have either of two interpretations. The virus may fail to reach the circulation or the presence of an established infection in the allantoic cavity in some way protects the embryo against any harmful effect of such virus as does reach it. Both possibilities were experimentally investigated.

A detailed study was made of twenty-four 11-day embryos inoculated with NWS (10^{-4}) in the allantoic cavity. When examined on the fourth day 10/12 showed evidence of infection, 9 with positive fluids and no embryo lesions, one with haemorrhagic lesions and no haemagglutinin. Titration of brains taken on the 2nd, 3rd and 4th days showed virus to be present in every one of the twelve tested. Three out of four tested on the second day, however, showed only minimal amounts. It is evident that NWS regularly reaches the circulation of the embryo and probably multiplies to some extent in the brain without producing haemorrhagic lesions in more than a small proportion.

The other approach was to induce an allantoic infection and, after an interval, to give an intravenous inoculation of NWS which would normally produce 100 p.c. of fatal haemorrhagic infections. Such experiments have consistently shown a striking protective effect which is equally or usually better shown when protective allantoic inoculation is made with MEL.

Three experiments will be described in which the intravenous challenge dose of NWS was successively increased from 10^{-6} to 10^{-5} and to 10^{-4}. In each experiment 11-day embryos were inoculated with appropriate dilutions of NWS or MEL into the allantoic cavity. Eighteen to twenty-two hours later an intravenous injection of NWS was given and after a further 3 days' incubation the eggs were opened and the embryos examined. Any embryos in which the intravenous inoculum was not clearly seen to enter the circulation were discarded. Table 4 shows the results of the first two experiments.

In a third experiment ten 11-day embryos were inoculated with MEL, 10^{-2}, into the allantoic cavity and ten with saline. Next day all were inoculated intravenously with NWS, 10^{-4}, and in a further three days all embryos were examined. Those receiving MEL showed 8 alive without lesions and 2 dead,

also without lesions. Of the controls given saline initially, one intravenous inoculation failed, the other nine all showed typical haemorrhagic brain lesions, 7 being dead and 2 being moribund.

TABLE 4.

Protection against intravenous NWS by an established allantoic infection.

Allantoic inoculum	Result of intravenous NWS, 10^{-6}			
	Specific death	Non-specific death	No lesion	Intravenous titration of NWS
NWS 10^{-5}	1	0	2	10^{-9} 0/4
10^{-4}	1	0	3	10^{-8} 0/4
10^{-3}	2	0	2	10^{-7} 3/4
10^{-2}	2	0	2	10^{-6} 4/4
MEL 10^{-6}	0	1	3	
10^{-4}	0	0	4	
10^{-2}	1	0	3	
Saline	4	0	0	
	intravenous NWS 10^{-5}			
MEL 10^{-6}	1	2	1	10^{-8} 3/5
10^{-4}	0	3	3	10^{-7} 3/5
10^{-2}	0	1	5	10^{-6} 5/5
10^{0}	0	1	3	10^{-5} 4/5
Saline	6	0	0	

Several attempts to carry out the reverse experiment to see whether the initiation of an infection intravenously would protect against multiplication of virus introduced subsequently into the allantoic cavity were unsuccessful. Major difficulties were infection of the allantoic cavity during intravenous inoculation and early death of the embryo. This aspect of the work will therefore not be discussed further here.

Experiments were made to determine whether NWS persisted in the brain after intravenous inoculation, in those embryos protected against brain lesions by the existence of an established MEL infection of the allantois. The brain emulsions taken 3 days after the intravenous inoculation were titrated in dilute anti-MEL serum. Three of the four examined contained NWS virus in one to a titre of 10^{-2}. One may conclude that the virus survives but that multiplication is very slight in these protected embryos.

The possible rôle of interference in the protective effect.

In the sense that the phenomenon which we have described is a protection against a normal damaging effect of one type of virus infection by the co-existence of another infection in the same host, it must be regarded as a form of interference. We are not aware, however, of any analogous example of interference shown by inoculation of the same virus by two different routes in the same host. The nearest corresponding observation is Magrassi's finding

that, under very strictly limited conditions, herpes encephalitis in rabbits may be prevented by simultaneous inoculation of the same strain on the cornea and intracerebrally (Magrassi, 1936).

It was necessary, therefore, to examine the conditions under which interference effects of the orthodox type could be demonstrated between MEL and NWS in the chick embryo.

If a mixture of MEL and NWS is inoculated into the allantoic cavity, the more vigorous MEL will almost always overgrow the NWS. For interference experiments inactivated MEL is therefore required. Stock MEL was heated in ampoules to the temperature shown and 0·15 ml. inoculated into the allantoic cavity of 11-day embryos. Next day NWS at 10^{-4} or 10^{-2} was inoculated into the same cavity. Four days later the presence of haemagglutinin in the fluid and of lesions in the embryo were checked. The results appear in Table 5.

TABLE 5.

Interference of NWS by inactivated MEL in the allantoic cavity.

1st MEL inoculum	2nd NWS inoculum	Result, in 4 eggs			
Heated to 100° C.	10^{-2}	+/—	—/+	—/—	N
	10^{-4}	+/—	+/—	—/+	—/—
30 mins. at 60° C.	10^{-2}	—/+	—/+	N	N
	10^{-4}	—/+	—/—	—/—	—/—
30 mins. at 57° C.	10^{-2}	—/+	—/+	N	N
	10^{-4}	—/—	—/—	—/—	N
30 mins. at 55° C.	10^{-2}	—/—	N	N	N
	10^{-4}	+/—	—/—	—/—	—/—
Unheated	10^{-2}	3 alive, normal			
	10^{-4}	3 alive, normal			
Nil	10^{-4}	As in Table 1			

+/— = fluid positive, no lesions in embryo
—/+ = fluid negative, specific lesions in embryo
+/+ = fluid positive, specific lesions in embryo
—/— = no evidence of infection
N = no evidence of infection, dead embryo

In brief, the non-infective MEL, heated between 55° C. and 60° C. reduced the incidence of positive fluids from the 15 to 20 expected to 1 out of 24 possible, but allowed 5 specific haemorrhagic deaths plus 8 deaths without haemorrhagic lesions, most of which were probably the result of virus multiplication. With boiled virus fluid, the results were equivalent to the normal findings with NWS. We may conclude that heat inactivated MEL virus effectively interferes with the multiplication of NWS in the allantoic cavity, but does not prevent passage of NWS to the chick embryo.

The possibility that direct interference could be demonstrated by consecutive intravenous inoculation of MEL and of NWS was explored in the follow-

ing experiment. Twenty 12-day embryos were candled to find two veins suitable for intravenous inoculation and drilled over both sites. Each received an intravenous inoculation of MEL, 10^{-4} and 5 hours later 10 were successfully inoculated with NWS, 10^{-4} into the second vein and 5 receiving only the first virus were reserved as controls. Table 6 contains the results obtained when the eggs were opened after three days' incubation. Despite the fact that, in this instance, all the embryos receiving MEL alone died in three days, the majority of those receiving an additional inoculum of NWS demonstrated the lesions characteristic of NWS infection.

TABLE 6.

Failure of interference by active MEL by the intravenous route.

Intravenous inocula	Specific deaths	Deaths without lesions
MEL 10^{-4} — NWS 10^{-4}	7	3
MEL 10^{-4}	0	5

Neither the allantoic nor the intravenous type of interference experiment offers any indication that orthodox interference processes are concerned in protecting the embryo against the haemorrhagic action of intravascular NWS.

DISCUSSION.

To a large extent the results reported in this paper are concerned with providing a background for subsequent studies of recombination in the chick embryo. Discussion here will, however, be confined to the phenomenon of prevention of haemorrhagic lesions in the embryo by co-existent allantoic infection with the same or with another virus. The phenomenon is striking and regularly reproducible with the strains that we have used, but it does not necessarily hold for all strains capable of causing haemorrhagic lesions when administered intravenously. We found it impossible, for instance, to protect against the lethal effect of MEL-Egg by a MEL infection in the allantoic cavity and when the challenge was a large dose of MEL the amount of "protection" was significant, but obscured by the large number of "non-specific" deaths in each series.

With this reservation, we can summarize the position as follows. When even a minimal dose of NWS reaches the embryo by the vascular route, a rapid multiplication ensues with production of specific haemorrhagic lesions and death. When, on the other hand, an infection of the allantoic cavity is already established at the time when virus reaches the embryo, haemorrhagic lesions are not produced and most embryos survive at least one or two extra days. The strain MEL never produces haemorrhagic damage except when given in large doses intravenously, though it can reach the embryo from other sites of inoculation

and, particularly in large inocula, causes death of the embryo without characteristic, naked eye lesions.

The haemorrhagic lesions must result primarily from damage to the vascular endothelium and, since the earliest stage consists of multiple minute haemorrhages, usually in the optic lobes of the brain, we can legitimately assume that it is essentially damage to the capillary endothelium that is responsible. It is natural to think that the damage results from virus multiplication in the endothelial cells and this is supported by the fact that large amounts of virus are present in the disorganized intracranial contents. The possibility that haemorrhages result from toxic products produced by virus multiplication elsewhere cannot be absolutely excluded, but in view of the failure of other types of lethal virus infection to produce the characteristic picture, it seems extremely unlikely. We may adopt as a working hypothesis that an invasive strain of influenza A virus is capable (a) of multiplication in the cells of the capillary endothelium and (b) of producing, as a result, sufficient structural damage to allow haemorrhage to occur. If this is so, the production of haemorrhagic lesions could possibly be inhibited by (1) failure of the cells to become infected, (2) inhibition of virus multiplication within the cells or (3) by failure of virus multiplication to produce sufficient structural damage to the cell.

The first possibility can be eliminated by the fact that small amounts of virus regularly reach the embryonic circulation and we must look for means by which one or both of the second and third possibilities may be mediated. The current interpretation of interference between influenza viruses (Henle, 1950; Isaacs and Edney, 1950; Fazekas and Cairns, 1952) is that the interfering virus, be it active or inactive, enters the cell and in some way renders key points within the cell inaccessible to the second challenge virus. Fazekas and Cairns (1952) have given quantitative data to show that a single heat-killed virus particle renders the cell which it enters completely refractory to a second infection. In the system which we are considering it seems quite impossible to conceive that sufficient MEL virus can enter the circulation from the allantoic cavity to block the great majority of all endothelial cells susceptible to attack by NWS virus. Homologous block by NWS itself could only be conceivable if the virus particles entering the circulation differed sharply from those inoculated and those which subsequently appeared in the allantoic fluid. Since the effect is apparent with small allantoic inocula which would not produce detectable amounts of "incomplete" virus, there is no available analogy and the suggestion may be discarded. It is in fact impossible to accept "interference" in its current sense as having anything to do with the phenomenon of protection against haemorrhagic lesions.

If virus as such is not concerned, we must assume that the effect of the allantoic infection on the vascular endothelium is governed by some component

other than virus in the circulating blood. The effect may be either positive or negative. Some product of virus-cell interaction may pass to the vascular endothelium and so modify its reactivity in the required fashion or the processes going on in the allantoic membrane may abstract from the circulation some component which is needed if the virus is to multiply in and damage the vascular endothelium. It is not easy to devise an experimental decision between the two types of hypothesis. Tissue extracts are highly toxic when given intravenously to the chick embryo and the impossibility of removing embryos uncontaminated by allantoic fluid, almost eliminates any experimental analysis along conventional lines. On general grounds we are attracted to the hypothesis that virus multiplication and associated processes provide a steady drain on some component of the blood which can be synthesized or made available only at a strictly limited rate. This component is assumed to be necessary for the complete virus-cell interaction. In a very early contribution to the study of interference between viruses Andrewes (1942) reached a similar conclusion in regard to the interference between two strains of influenza virus in tissue culture.

There is also another situation in which the same component may perhaps be involved. This is in relation to the formation of "incomplete" virus. With a suitable strain, e.g. PR8, the ratio of infectivity to haemagglutinin titre of harvested allantoic fluids depends on the size of the initial inoculum (von Magnus, 1949). With a small inoculum such as 10^{-6}, a fluid harvested when the peak of virus production has just been attained has a ratio indicating to a close approximation that all the virus particles are both agglutinating and infective. If the inoculum is of undiluted virus, the yield has characters suggesting that only 1/100 of the agglutinating particles are infective. Cairns and Edney's (1952) finding that the production of incomplete virus is not a result of multiple infection and that it appears simultaneously with complete virus suggests strongly that the incomplete virus is produced in the first cycle of infection because of the lack of some necessary component supplied by the blood-stream. The production of uniformly infective virus from a small inoculum, that is after several cycles of infection and liberation of virus, must mean that the capacity to synthesize or make available the component can be increased gradually as a result of some phase of the process of allantoic infection.

It is at least a possibility that the inadequacy of an unknown component "X" might be responsible for the production of predominantly incomplete virus in the infected allantoic cell and for the failure of infection induced in an endothelial cell to proceed to structural damage. Theoretically at least it should be possible to identify the hypothetical compound by a search for substances which, when administered intravenously, or perhaps into the yolk sac, would allow production of complete virus from a large seed inoculum and would annul the protective effect of an existing allantoic infection against NWS intravenously.

SUMMARY.

The influenza A strain NWS only rarely produces haemorrhagic lesions in the embryo after allantoic infection although infection by the chorioallantoic and intravenous routes regularly produces such lesions.

Influenza A, strain MEL, produces haemorrhagic brains only when relatively large intravenous inocula are used. Infection by other routes results in passage to the embryo, but no haemorrhagic lesions are produced.

If an allantoic infection with MEL or NWS is induced 18 to 20 hours before intravenous inoculation of NWS, the latter does not produce haemorrhagic lesions in the embryo.

The working hypothesis is presented that the protection is mediated by the withdrawal from circulation of a limiting component necessary for complete virus-cell interaction.

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II. GENETIC INTERACTION BETWEEN INFLUENZA VIRUS STRAINS IN THE CHICK EMBRYO

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The basis of the claim made by Burnet and Lind (1951) that recombinants could be recovered from the brains of mice doubly inoculated with the strains MEL and NWS was the consistent isolation of strains called by them N-M (neuro-MEL) strains. These have several characteristics which distinguish them from either of the parent strains or from any possible mixture of these. They are serologically MEL and the haemagglutinin is not destroyed by heating to 55° C. as is the case with NWS. Unlike MEL they are converted to the indicator phase by simple heating and are regularly invasive for the chick embryo producing haemorrhagic brain and muscle lesions after chorioallantoic inoculation. Many such strains produce fatal encephalitis in mice on intracerebral inoculation but by no means all (Burnet and Edney, 1951). Although the name N-M originally signified the possession of pathogenicity for the mouse brain it has now been taken as a convenient name for strains showing the characteristic *in vitro* behaviour irrespective of their range of pathogenicity.

In the mouse experiments such N-M strains are most readily obtained on the fourth day after injection of the mixed inoculum. It is usual to obtain one or both of the original MEL and NWS types as well. The strains isolated on the fourth day are obviously the successful survivors of a whole series of infective generations in the brain cells. Detailed analysis of the situation would be difficult or impossible and a simpler and more controllable experimental system was sought.

The immediate objectives were to establish two of the implications of the hypothesis that N-Ms arose by genetic recombination between the original types. If this were the case the recombinants should be detectable whenever cells were infected simultaneously by both parental types, i.e. at any time after a single cycle of cell infection had been completed. Secondly, if interaction between two doubly "labelled" viruses AB and ab gives a proportion of Ab forms the

[1] This work was carried out while in receipt of a grant from the Nuffield Foundation.

reciprocal recombinant aB should also be obtained. In other words, from the MEL-NWS system not only an N-M type but also a strain serologically (WS) but lacking neurotropism and invasiveness for the chick embryo should be obtainable. The second and third papers of the present series are concerned with recording the progress that has been made toward these objectives.

In this second paper we shall deal with the production of invasive (and often neurotropic) strains serologically MEL from mixed infections with NWS and MEL in the chick embryo. In all respects these strains are similar to the N-Ms of previous papers and will be so referred to in this.

Materials and Methods.

The virus strains MEL and NWS were the same as those described in the first paper of this series and they were stored and handled in the same fashion. Other techniques described there are applicable to the present work. Additional methods needing description are as follows:

Indicator state. A virus is said to be converted to the indicator state by heating or other manipulation when its haemagglutinating power is retained but has become susceptible to inhibition by high dilutions of suitable mucoid inhibitors. In the test used allantoic fluid was heated at 56° C. for 30 minutes, titrated and 5 agglutinating doses (AD) tested against an equal volume of serial ten-fold dilutions of inhibitor. Ovomucin as prepared by Gottschalk and Lind was used in most of the work but in the later stages a semipurified preparation from meconium was found more convenient. The results with the two were identical and were controlled by including heated LEE virus in each series of tests.

"One-cycle" allantoic fluids. The preparation of fluids containing the virus produced by only one cycle of reproduction has been described by Cairns (1952). With the difference that, in the present work, infection was produced by two viruses simultaneously, the technique used is basically that of Cairns. Two holes are drilled in 11-day embryonated eggs as for sub-allantoic inoculation. Through one 0·05 ml. of undiluted NWS seed virus and 0·05 ml. of MEL seed virus diluted 1: 10 are introduced simultaneously. Three hours' incubation at 35° C. are allowed for absorption of seed viruses, then 1,500 units of RDE (receptor-destroying enzyme) are inoculated and incubation continued for another period of 1 hour. The action of the RDE removes excess virus which is absorbed to the surface of the allantoic membrane and minimizes the amount of absorption of virus produced at the end of the first reproductive cycle by destroying receptors of any cells which are still uninfected. As we have found from experience that virus haemagglutinin may appear fairly rapidly in the allantoic fluid after the inoculation of excessively large doses of infective virus, the whole contents of the allantoic cavity are removed through the second drill-hole at 4 hours after the primary inoculation and the cavity thoroughly flushed with four successive volumes of 5 ml. of warm normal saline. Finally the fluid is replaced by 5 ml. of normal horse serum saline warmed to 37° C. and incubation continued to the 8th hour after infection. At this time the contents of the allantoic cavities may be harvested or the eggs may be rapidly chilled to below 4° C. and harvested on the following morning after storage at 4° C. overnight. The fluids are referred to as "one-cycle" fluids and must obviously contain, in addition to a relatively small amount of the original inoculum, virus produced in one reproductive cycle from cells infected with NWS only, with MEL only and from cells infected with both NWS and MEL. A fraction of the haemagglutinin produced may be non-infective, that is incomplete virus (von Magnus, 1949).

EXPERIMENTAL.

Experiments using chorioallantoic inoculation.

The work reported in the preceding paper (Burnet and Fraser, 1952) on the invasion of the embryo by NWS and MEL had indicated that the allantoic cavity would be an unsatisfactory medium for recombination experiments. In the first place any other type that might be present would be rapidly overgrown by MEL, secondly the inhibitory effect of an established infection on the manifestations of an invasive strain in the embryo would eliminate the possibility of using haemorrhagic lesions as evidence of the presence of invasive types. This effect was not shown on the chorioallantois and it was already known that all N-M strains from mouse brain experiments were capable of invading the embryo and producing haemorrhagic lesions after chorioallantoic inoculation. The first experiments with double inoculations were therefore made by this route and it soon became apparent that the virus which could be recovered from the haemorrhagic brain lesions was frequently of N-M type.

Four experiments were conducted on these lines from all of which N-M strains were recovered. The first of these is described here as a type experiment.

A mixture containing equal parts of MEL undiluted and of NWS diluted 1 : 100 was inoculated on to the chorioallantoic membrane of six 12-day embryos. Of four opened at the third day of incubation, one had early haemorrhagic lesions in the brain (81035). On the fourth day the remaining two embryos died with grossly haemorrhagic lesions (81036 and 81037). The three brains were emulsified and titrated by suballantoic inoculation and fluids from the highest positive dilutions characterized.

TABLE 1.

The isolation of N-M strain by direct titration of emulsions of chick embryo brains.

Embryo brain	Dilution of inoculum			
	10^{-4}	10^{-3}	10^{-2}	10^{0}
81035	N-M, N-M,— —	4/4	3/4	3/4
81036	0/4	0/4	M, M, NWS, +	4/4
81037	0/4	0/4	M, M, M, +	4/4

4/4 = 4 positive fluids/4 embryos inoculated
\+ = positive fluid, not identified
— = negative fluid
N-M, M(MEL), NWS = types of virus when identified.

As is shown in Table 1, two fluids 81104 and 81105 had N-M characteristics. The emulsion of brain 81035, which had been put aside in a dry-ice cabinet, was inoculated in a second test into the allantoic cavity of 14 11-day embryos. Of ten positive fluids, nine were characterized; three were N-M,

numbers 81245, 81246 and 81247; 4 were M and 2 were NWS. These fluids and fluids from limiting positive dilutions of a further titration of fluids 81104 and 81105 were tested for intracerebral pathogenicity in mice. Table 2 shows the character of the original and of the derived allantoic fluids.

TABLE 2.

The characteristics of N-M fluids obtained from embryo brain 81035

Infective fluid	Haemagglutinin: Unheated	Haemagglutinin: Heated 55° C.	Serological type	Indicator titre	*In vitro* type	Pathogenicity to mice
81104 original	1280	480	MEL	$10^{-4\cdot5}$	N-M	
„ at 10^{-9}, 81368	180	120	MEL	$10^{-4\cdot5}$	N-M	
„ at 10^{-8}, 81370	480	240	MEL	10^{-5}	N-M	5,5*,5,5
„ at 10^{-8}, 81372	540	240	MEL	10^{-5}	N-M	
81105 original	200	200	MEL	$10^{-4\cdot5}$	N-M	
„ at 10^{-8}, 81232	80	30	MEL	$10^{-4\cdot5}$	N-M	6,7,S,S
„ at 10^{-8}, 81234	480	480	MEL	$10^{-4\cdot5}$	N-M	
81245 original	70	70	MEL	$10^{-4\cdot5}$	N-M	S,S,S,S
81246 original	160	60	MEL	10^{-5}	N-M	5,5*,6,6
81251 original	40	15	MEL	10^{-5}	N-M	S,S,S,S

Under pathogenicity to mice: Figures indicate day of death after intracerebral inoculation. S = survived. * N-M recovered from brain.

Brains from two mice which had encephalitis, indicated in the Table by an asterisk, produced N-Ms on subinoculation at limiting positive dilution. To sum up, five isolations of N-M were made from embryo brain 81035, which contained both NWS and MEL in about the same quantity. Of these fluids, two produced regularly fatal infection in mice inoculated intracerebrally, one produced death only in a proportion of the mice and two did not produce symptoms.

Three other experiments along the same general lines each gave strains with N-M characters *in vitro*. Four strains from two experiments were tested in mice by intracerebral inoculation. Two produced fatal encephalitis in all mice, two had no evident effect. From all the experiments therefore nine N-M strains were obtained all producing haemorrhagic lesions in the chick embryo. Four had full neuropathogenicity, one was of intermediate virulence and four were avirulent by the intracerebral route in mice.

This demonstration of the recovery of N-M strains after double infection on the chorioallantois gave no indication of where their production took place but made it clear that the chick embryo could provide appropriate conditions for their appearance. Attention was therefore turned to the allantoic cavity with the object of using the chorioallantoic technique to determine what took place in the early stages of allantoic infection. Our working hypothesis that N-M strains were produced as the result of a process of recombination in cells simultaneously infected with both "parent" viruses, would imply that this might take place in any type of susceptible cell. The process of influenza

virus multiplication has been much more intensively studied in the allantoic cavity than elsewhere and the most convenient way of producing massive double infection was obviously to inoculate large amounts of virus into the allantoic cavity. Equally if the product of double infection was to be obtained before its composition could be modified by competitive multiplication and survival the fluid should be harvested as soon as one cycle of infection had been completed.

Experiments with "one-cycle" fluids after mixed infection in the allantoic cavity.

The method of preparing one-cycle fluids developed by Cairns (1952) is given in detail under methods. Briefly it involves inoculation into the allantoic cavity of amounts of each virus MEL and NWS large enough to ensure that double infection of cells will be a frequent occurrence. The use of RDE and subsequent flushing of the allantoic cavity with saline were designed first to ensure as thorough removal as possible of all virus in the original inoculum which had not entered the cells, and second to hinder adsorption of the virus liberated in the first cycle to previously uninfected cells. Subsequent experience suggests that these aims can only be incompletely achieved. It is difficult to wash and drain all parts of the complex allantoic cavity and NWS, as has been noted by Cairns, is abnormally low in its infectivity/haemagglutinin ratio so that the amount taken up in the time allowed is probably unduly low.

Most of the experiments to be discussed were based on a single pool 82710 produced as described under methods. This one-cycle fluid by direct infectivity titration showed a titre of $10^{6.5}$, the fluids produced near the end point having the normal characters of MEL. When titrated on the chorioallantois in the presence of anti-NWS serum, haemorrhagic lesions due to N-M virus were obtained. The problem presented therefore was to determine the constitution of fluid 82710, i.e. the concentration of MEL and NWS virus and whether N-M virus was actually present in the fluid or had developed after subinoculation on the chorioallantois.

The most practicable approach was to use antisera to inhibit one or other component. Provided the concentration of antiserum was kept low enough to have no action on the heterologous virus and that there was not too great an excess of the homologous virus, this allowed a decision as to the amount of MEL and NWS present.

In Table 3 are combined the results of titrations in the allantoic cavity of pure strains of the various types of virus concerned in the present work and of the first cycle fluid 82710. In addition to direct titrations with dilutions in normal horse serum saline, each virus has also been titrated in anti-MEL and anti-NWS sera, the dilutions of virus being made in 1 : 50 antiserum. This provides a background to the use made of titration in immune serum at various stages of this work.

TABLE 3.

Infectivity titrations of MEL, NWS and N-M and of the one-cycle fluid 82710 in normal serum saline and in dilute antisera.

Dilution medium	Infectivity titre—allantoic route				Infectivity titre-chorioallantoic route
	MEL	NWS	N-M	Fl. 82710	NWS
Normal serum saline	$10^{8.5}$	$10^{6.0}$	$10^{8.7}$	$10^{6.5}$	$10^{7.5}$
Serum 359 1: 50 Anti-MEL	$10^{5.0}$	$10^{6.5}$	$10^{2.5}$	$10^{2.5}$	NT
Serum 375 1: 50 Anti-NWS	$10^{8.8}$	$10^{2.5}$	$10^{8.8}$	$10^{6.5}$	$10^{4.0}$

NT = Not tested.

From these results we deduce that fluid 82710 has a content of MEL which we express as MEL $10^{6.5}$, i.e. each 0·05 ml. contains $10^{6.5}$ ID_{50}. The maximum possible amount of NWS is $10^{2.5}$. An alternative method of titrating NWS is to use the chorioallantois and take the presence of haemorrhagic lesions in the embryo as index of infection with NWS. On this basis results were not fully consistent but suggested a value of 10^{2}. When, however, the chorioallantoic titration was carried out in anti-NWS serum, 2 of 4 embryos inoculated at 10^{-1} showed haemorrhagic lesions from which *N-Ms* were isolated and characterized.

There was therefore an *a priori* case for assuming that the fluid 82710 was composed approximately as follows: MEL $10^{6.5}$, NWS 10^{2}, N-M 10^{1}.

In order to establish the existence of a small N-M content with certainty it was necessary to show first that an artificial mixture of MEL and NWS placed on the chorioallantois with the standard amount of anti-NWS serum did not produce N-M lesions in the embryo and second that no NWS virus could be isolated from the brains which contained N-M after chorioallantoic inoculation of 82710 plus anti-NWS serum. The behaviour of artificial mixtures of MEL and NWS virus on the chorioallantois with and without anti-NWS can be shown by combining the results of three experiments in Table 4. In all cases MEL virus was present in amounts around 10^{6} while the NWS content varied as shown, but was always greater than that present in fluid 82710.

TABLE 4.

Results of inoculating MEL-NWS mixtures on the chorioallantois.

Inoculum		Incidence of specific lesions in embryos	
MEL	NWS	Dilution in normal serum saline	Dilution in anti-NWS serum 1: 50
10^{5}	$10^{4.5}$	3/4	1/10
$10^{6.5}$	$10^{3.0}$	10/11	0/9
$10^{6.5}$	$10^{4.5}$	...	1/11

Number of embryos showing haemorrhagic lesions/number inoculated.

By contrast Table 5 shows the results of similar experiments done in parallel with those of Table 4 in which two first-cycle fluids 82710 and another 83892 from an experiment to be mentioned later which had virtually identical content of MEL and NWS were tested. At the same time an artificial mixture was prepared of appropriate amounts of allantoic fluids infected with pure clones of the three virus types to have a composition MEL $10^{6.5}$ NWS $10^{3.0}$ N-M $10^{2.0}$ and tested similarly.

TABLE 5.

Results of inoculating one cycle fluids and a corresponding artificial mixture on the chorioallantois.

Inoculum	Incidence of specific lesions in embryos	
	Normal serum saline	Anti-NWS serum 1: 50
Fluid 82710	9/10	6/11
Fluid 83892	4/4	6/12
Mixture MEL $10^{6.5}$ NWS $10^{3.0}$ N-M $10^{2.0}$	...	7/12

There is a very clear distinction between the two sets of results and in view of the neutralizing capacity of the serum used (F 373 at 1:50) we can be certain that the N-Ms derived from the first cycle fluids were actually present in those fluids and were not subsequently produced in the embryos from whose brains they were isolated.

In view of suggestions that N-Ms may be merely pre-existent mutants of MEL present but undemonstrable by ordinary techniques in all allantoic fluid virus populations, the sharp difference in the behaviour of mixed viruses with and without a small addition of N-M should be stressed.

As a further means of establishing the existence of N-M particles in the first-cycle fluid efforts were made to find NWS in the brains of embryos developing haemorrhagic lesions after inoculation on the chorioallantois in the presence of anti-NWS serum. This was needed to counter the objection that the origin of the N-Ms in these brains might be attributable to interaction there of MEL and small amounts of NWS escaping the antiserum. In such circumstances there would undoubtedly be some NWS as well as N-M in the brain emulsion. However, in addition to showing only N-M at limit dilutions of allantoic titrations, no NWS could be obtained when the same brain emulsions were titrated in anti-MEL serum which strongly inhibited N-Ms. That the method is practicable for the purpose was shown by the isolation of NWS from brains with haemorrhagic lesions after inoculation without antiserum.

A complete repetition of the essential features of the above experiments was carried out using new stocks of MEL and NWS reisolated from limit dilution. Using the same technique six one-cycle fluids were harvested and tested separately. From five of these N-M strains were readily isolated but when

the haemorrhagic brain lesions were tested in anti-MEL serum four showed the presence of NWS or the modified form NWS[1] described by Burnet (1951). Detailed study of three of these one-cycle fluids showed that by using an increased amount (1:25) of NWS antiserum, two produced infections with N-Ms only present in the brain.

TABLE 6.

Chorioallantoic titration of 3 one-cycle fluids in anti-NWS serum, showing incidence of lesions and type of causative virus.

Fluid (with NWS titre)	In horse serum saline fluid at 1:100	In antiserum 1:50 fluid at 1:1	In antiserum 1:25 fluid at 1:1	1:3	1:10	1:100
83890 (10^{-4})	4/4 NWS and N-M	2/4 NWS and N-M	3/3 NWS and N-M	0/4 —	2/4 NWS and N-M	2/4 N-M
83892 ($<10^{-3}$)	4/4 N-M (2 specs. lost)	3/4 NWS and N-M	2/4 N-M	3/4 N-M	3/4 N-M	1/4 N-M
83893 ($<10^{-3}$)	2/3 NWS and N-M	3/4 NWS and N-M	2/4 N-M	2/4 N-M	2/4 N-M	0/4 —

NWS and N-M indicate types of virus recovered from haemorrhagic brains. They do not indicate the frequency of isolation of each strain. N-M predominated in all cases.

As a final control four one-cycle fluids were prepared using MEL alone. These tested in similar fashion on the chorioallantois with anti-NWS serum gave no haemorrhagic lesions in any of the 24 embryos used.

DISCUSSION.

Work in this laboratory over the past 3 years has made it clear that N-M strains as defined earlier can be readily obtained from mixed infections with NWS and MEL either in the mouse or the chick embryo. The facts are simple and definite but the interpretation by Burnet and Lind (1951) that the N-M strains arose by a process of recombination has not so far been accepted by virologists generally.

The alternative possibilities that have been suggested in informal discussion are (1) that the N-M strains are mutants of MEL occurring spontaneously but masked by interference except under special circumstances. The possibility that they are mutants of NWS should perhaps also be formally considered.

(2) That they result from an environmental influence by which the NWS-induced tissue damage confers a new invasive or neurotropic quality on a proportion of MEL virus particles.

(3) That a process analogous to the pneumococcal type transformation is occurring, a transforming principle derived from NWS passing to MEL and inducing an inheritable change in N-M.

At the virus level the last hypothesis is almost the same as that of recombination and this would be an inappropriate place to discuss what differences there are between the two hypotheses.

We feel, however, that the present work allows the rejection of hypotheses 1 and 2 and the evidence against the view that N-Ms arise by mutation or by environmental influence may be briefly stated. The most direct evidence against mutation is that normal or first-cycle fluids prepared from MEL alone never give demonstrable N-M strains when handled by methods which allow their ready isolation from first-cycle fluids from mixed infections or from artificial mixtures to which small amounts of known N-M have been added. Further, it has been shown incidentally to the present work that large excess of MEL does not produce interference against N-M on the chorioallantoic membrane and if N-Ms were regularly occurring mutants it is inconceivable that MEL inoculations on the chorioallantois should never produce haemorrhagic lesions in the embryo. In a large experience we have never found such lesions.

By environmental influence we would imply any means by which the growth of a virus strain in an environment favourable to itself may endow a similar capacity to flourish in that environment on another introduced strain not normally capable of doing so. If our evidence is sound that we have shown N-Ms to be produced in the first cycle of infection in the allantoic cavity any hypothesis of this sort is invalidated. N-Ms grow *less* readily than MEL in the allantoic cavity and there is surely no conceivable reason why "adaptation" to allantoic growth should confer capacity to produce encephalitis in the mouse.

The application of the approach used in this paper to a slightly different system has provided additional evidence which makes it advisable to postpone discussion of the remaining hypotheses until the new experimental work is presented in the third paper of this series.

SUMMARY.

Mixed infection with the influenza A strains MEL and NWS on the chorioallantois gives rise to haemorrhagic brain lesions from which "recombinant" N-M strains can be isolated which combine the serological character MEL with the characteristic neurotropic and invasive qualities of NWS.

"One-cycle fluids" produced by mixed infection with NWS and MEL in the allantoic cavity have been analysed by their behaviour with and without antisera on the chorioallantois. Evidence is provided that N-M forms are produced in the first cycle of infection in doubly infected allantoic cells.

It is concluded that N-M forms can be produced only by some type of genetic interaction between MEL and NWS viruses.

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STUDIES ON RECOMBINATION WITH INFLUENZA VIRUSES IN THE CHICK EMBRYO

III. RECIPROCAL GENETIC INTERACTION BETWEEN TWO INFLUENZA VIRUS STRAINS

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In an earlier paper (Burnet and Lind, 1951) we adopted the working hypothesis that influenza virus behaves similarly to the T-even group of bacteriophages and when double infection of the host cell occurs genetic recombination can occur between the two invading types. The technical methods of handling bacterial virus experiments are remote from those necessary with influenza virus studies and in particular there is no technique in sight for obtaining the progeny of a single animal cell infected by two distinct virus types. Nevertheless, there has been sufficient work done on the multiplication of influenza virus in the allantoic cavity to allow a forecast of the conditions necessary to obtain the maximal yield of recombinants. The requirements are: (1) The introduction of the largest practical amount of fresh virus into the allantoic cavity both components being present in equivalent amounts. (2) The two types used should have approximately equal growth characteristics and reach similar final titres in the allantoic cavity. (3) After a period has been allowed for entry of virus into the cells all residual virus should be removed by a combination of repeated washing and treatment with receptor destroying enzyme RDE. (4) The virus liberated in the first cycle should be collected: RDE treatment will prevent adsorption of this to the allantoic cells and ensure a harvest representative of the viable virus liberated from the cells. (5) The two viruses used should be of the same species but should differ by as wide a range of marker characteristics as possible.

This formulation is very largely based on the method recently used by Cairns (1952) to estimate the virus liberated after infection of a single cell in the allantoic cavity.

In the preceding paper the NWS-MEL system was used and N-M forms were regularly obtained but never in such numbers as to allow direct demonstration by simple dilution of the one-cycle fluid. However, the experience gained plus a growing realization of the principles stated above led to the substitution of NWS by the closely related WSE strain which differs only (a)

in having no pathogenicity for mice on intracerebral injection; (b) in growing much more freely in the allantoic cavity and (c) in having a higher pathogenicity for mice by the intranasal route.

The use of the combination MEL-WSE given in the largest practicable dose was remarkably successful, analysis of the one-cycle fluid by simple limit titration showing not only forms equivalent to the N-M strains of the previous experiments but also even larger numbers of the long sought reciprocal recombinant. In view of the fact that WSE and all the derivatives with which we are concerned have no neuropathogenicity for mice the term N-M is not appropriate and we call the corresponding types in these experiments M+ in view of their development of the indicator state on simple heating. The serologically WS variant which does not become an indicator on simple heating is referred to as WS—.

MATERIALS AND METHODS.

Only matters not dealt with in the two preceding papers will be mentioned here.

Strain WSE. This derivative of the type influenza A virus WS (Smith, Andrewes and Laidlaw, 1933) was developed by chorioallantoic passage in 1937 (Burnet, 1949) and since 1940 has been maintained by occasional allantoic passage with intermediate storage in a dry ice refrigerator.

Intranasal inoculation of mice. In all cases the freshly-harvested fluid was diluted to a level corresponding to one haemagglutinating dose and 0·05 ml. administered from a calibrated Pasteur pipette to anaesthetized mice 4–5 weeks old. Surviving mice were killed at 7 days and the lung lesions given values from 0 to 4 according to the usual convention. Death on 6th or 7th day is given a value of 4, on the 3rd to 5th day a value of 5.

Inhibitor tests. In the standard test for indicator state, the fluid was heated 30 minutes at 56° C., its HA titre was determined and stock 5 AD dilution prepared. This was tested against equal volumes of 10-fold dilutions of a semi-purified meconium inhibitor supplied by Mr. C. C. Curtain (French, Curtain and Pye, in preparation). A stock solution of this material in 50 p.c. glycerol-saline kept well and provided a more convenient reagent than the ovomucin previously used. In the Tables the dilution of the glycerolated stock giving end point inhibition of 5 ADs is given logarithmically; heated LEE virus used as a standard gave a value of 3·5 (3,000).

TABLE 1.

Behaviour of standard indicator virus preparations with the three inhibitors used.

Indicator virus	Meconium	Meconium/WSE	Sheep mucoid
T.MEL	4	3·3	<2
H.WSE	4	<2	4·5
H.LEE	3·5	3·0	4

Figures give $\log_{10}$ dilution of stock inhibitor showing + end-point.

It was known that salivary gland mucoid from the sheep (McCrea, 1951; Burnet, 1948) acted strongly against derivatives of LEE and WSE in the indicator phase but had no action whatever against MEL derivatives which were readily inhibited by meconium, ovomucin

or other inhibitors. A glycerol-saline stock solution of material prepared by Dr. J. F. McCrea was used similarly to meconium for testing the reactivity of the strains being studied. Heated LEE gave a value of 4.

In a few experiments meconium inhibitor treated with WSE was used. Overnight action of the virus at 37° C. in the presence of Ca followed by inactivation of the virus by brief heating to 90° C. gave an inhibitor active against MEL and LEE indicator viruses to 3·0–3·3 but quite inactive against heated WSE. The action of these three inhibitors against the three standard indicator viruses T.MEL H.LEE H.WSE is as shown in Table 1.

One-cycle fluid preparation. The changes from the technique described in the second paper were to use as inoculum, virus freshly harvested from eggs inoculated 2 days previously, and to give a total volume of 5 ml. of mixed inoculum, adjusted to equivalent haemagglutinin titre of both types, after removal of an equal amount of allantoic fluid. Twelve-day embryos proved more convenient to handle than 11-day but it was noticed that with improving technique in washing out the allantoic cavity a number of embryos were dead at the completion of the manipulations and in later experiments warm Ringer solution was used for washing instead of saline.

EXPERIMENTAL.

A preliminary experiment in which the one-cycle fluid 85580 had an HA titre of 120, gave 5/6 infections at 10^{-6}, none at 10^{-7} or 10^{-8}. The five fluids by *in vitro* test included two of the type M+, one MEL, one atypical WS, and one subsequently shown to be a mixture of MEL and WS—.

With this as a basis the main experiment to be reported was then carried out using the same general procedure with minor refinements of timetable. The RDE was added at 1½ hours and washing commenced at 2¼ hours and completed (6 replacements) by 4 hours. The haemagglutinin titres at the beginning and at the end of the washings were for separate eggs 160-10.

Samples taken at 5½ hours showed HA titres between 80 and 160 and the fluids were drained 6-6½ hours after inoculation, i.e. well within the limits of the first cycle of infection. The fluids from 4 eggs were pooled and ampouled for storage in the dry ice refrigerator. A preliminary titration in the allantoic cavity using 6 eggs per 10-fold dilution was used as a sighting experiment and a more detailed titration with 20 eggs per dilution carried out at 10^{-6}, $10^{-6.5}$ and 10^{-7}. The preliminary titration of one-cycle fluid 85678 gave 6/6 positive fluids at 10^{-6}, 1/6 at 10^{-7} and none at higher dilutions. The repetition with larger numbers of eggs gave

10^{-6} 14/17 $10^{-6.5}$ 12/18 10^{-7} 3/18.

In vitro tests of all positive fluids for serological character MEL or WS and for conversion to indicator on simple heating allowed the following provisional classification of the 35 positive fluids that were studied from this experiment.

10^{-6}	11 MEL	4 M+	1 WSE	4 WS—	
$10^{-6.5}$	4 MEL	1 M+	1 WSE	3 WS—	2 indeterminate
10^{-7}	4 MEL				

Main attention was naturally concentrated on the two new types and these fluids were titrated and limit dilution fluids characterized. The majority bred true but several gave evidence that the original fluid had been a mixture. The two indeterminate fluids were mixtures of almost equal parts of MEL and WS—. Four other fluids gave evidence one way or another of having two components, the mixtures being MEL—M+, M+—WSE, M+—WS— and WSE—WS—. It is also not excluded that some other primary fluids may have had another unrecognized component present. Allowing for the additional components recognized in mixed fluids the overall yield was 22 MEL, 9 WS—, 6 M+ and 4 WSE strains. Since this order is also the order in which the viruses tend to dominate in mixed culture we find it strongly suggested that when virtually every cell is doubly infected with MEL and WSE nearly equal amounts of these four types are liberated. It must be stressed that no selective measure whatever was used, the one-cycle fluid was simply diluted to its limit of infectivity and all viruses capable of multiplying sufficiently to produce demonstrable haemagglutin in 3 days enumerated. Making a reasonable allowance for masking of one strain by another the ratio of viable virus particles present might be close to 4:2:2:1 for MEL, WS—, M+ and WSE respectively but if the intrinsic infectivity of the four types shows only minor differences it might equally be 1:1:1:1.

The characteristics of the two recombinant strains.

The two standard strains MEL and WSE are both influenza A viruses with many characteristics in common. In genetic work, however, we are only concerned with the differences between them and for our present purposes we have taken six pairs of characters as genetic markers for the two forms. These may be enumerated as follows:

(A) *Serological character* as tested either by inhibition of haemagglutination or inactivation in infectivity tests. In H.I. tests there is a slight cross-over with immune ferret sera but the difference between the two is so marked that there has never been the slightest doubt as to the serological type of any pure clone virus. With the sera actually used the homologous and heterologous titres against 5 ADs of virus were MEL antiserum, 1,600-100, NWS antiserum, 1,000-<100.

(B) *Heat stability.* If MEL allantoic fluid is heated for 30 minutes at 62° C. there is only a moderate fall in haemagglutinin titre; under the same conditions WSE haemagglutinin is completely inactivated.

(C) *Conversion to indicator state by simple heating.* MEL virus heated to 56° C. for 30 minutes as unmodified allantoic fluid shows no change in the behaviour of its HA against standard mucoid inhibitors. The haemagglutinating activity of heated WSE is inhibited by high dilutions of mucoid inhibitor

such as ovomucin or the semi-purified meconium inhibitor used in most of our experiments. The difference between the two strains is almost absolute.

(D) *Reactivity of heated virus with sheep salivary mucoid.* The inhibitor obtained by McCrea (1951) from the submandibular salivary gland of the sheep is unusual in having no inhibitory action against MEL even when the virus has been converted into a form very susceptible to inhibition by other mucoid inhibitors. This lack of action is almost limited to MEL, indicator preparations of LEE, WSE and PR8 are all inhibited in normal fashion.

(E) *Invasion of the chick embryo from the chorioallantois.* MEL infections on the chorioallantois allow some virus to reach the embryo and heavy infections very often kill the embryo by the fourth day but haemorrhagic lesions are never produced. WSE on the other hand always kills the embryo with gross haemorrhagic lesions in muscles, brain and other organs. From the lungs of such embryos it is usual to be able to extract enough virus to allow its type to be diagnosed by antihaemagglutinin tests.

(F) *Production of pneumonic lesions in mice inoculated intranasally.* The two strains we have used differ sharply in the effect produced when virus diluted to the 1 AD level is inoculated intranasally. Mice given WSE die with complete lung consolidation 4 to 6 days after inoculation, mice given MEL survive and show lesions involving less than half the lung surface.

It will be convenient to adopt the convention that in regard to these 6 differentiating points MEL has the character ABCDEF while WSE is represented by abcdef. This will allow a compact description of the other types of virus produced by their interaction. With minor reservations to be mentioned later all the strains isolated fall into 4 patterns only, the two originals ABCDEF, abcdef, M+ with the formula ABcDeF and WS— with the reciprocal formula abCdEf.

The serological type has always been unequivocal and no protocols need be included. More work needs to be done on the question of heat stability and only limited use has been made of heating at 62° C. in characterization of strains. Resistance to inactivation of haemagglutinin has always been associated with MEL serological character. On the other hand, if one tabulates the fall in haemagglutinin titre associated with 30 minutes' heating at 56° C. done primarily for determination of the indicator state the trend is distinctly different. There are individual discrepancies with all types but the order of increasing heat susceptibility is clearly MEL> WS—> M+> WSE, suggesting the existence of a minor transferable character. As we have not found a way of making the test reliable for the characterization of a given fluid it is not further considered.

Four strains each of M+ and WS— were twice purified by titration to limiting infective dilution and each L.I.D. fluid tested. The results shown in

Table 1 in regard to behaviour against mucoid inhibitors (characters Cc and Dd) have been quite consistent. This is almost necessarily the case as the primary differentiation into 4 types is based on the combination of serological type and reactivity of heated virus with meconium inhibitor. As already stressed sheep mucoid never reacts, in our experience, with any MEL derivative.

The findings on the chorioallantois factor (Ee) are somewhat variable and as was the case with the N-M derivatives of MEL+NWS the particular type of virulence concerned seems to be capable of expression at several degrees. MEL strains never give haemorrhagic lesions but some seem to give a higher proportion of deaths without lesions than others. WSE strains always kill with gross haemorrhagic lesions the only deviation being an occasional underdeveloped embryo which is killed early and is autolysed when examined. M+ strains are definitely variable. Five original fluids gave specific haemorrhagic deaths. Two gave at 4 days two living and one dead embryo all without lesions. One of the latter strain (85782) maintained its ABcDEF character on two limit dilution passages. Of the 4 typical fluids passaged at L.I.D. 3 remained capable of producing haemorrhagic lesions, the other 86118 had lost the power. It may be noted here that none of the M+ strains nor of the other 3 types had any power to produce encephalitic symptoms on intracerebral inoculation in mice. The WS— strains were consistently incapable of producing haemorrhagic lesions in chick embryos. The proportion of deaths varied from one test to another but no reproducible differences between strains could be recognized.

In the experiments with which we are concerned in this paper the degree of virulence for mice was remarkably constant in the sense that of the mice inoculated with 1 AD of WSE or WS— strains there were none that survived 7 days while of those given the same dose of MEL or M+ there were none which failed to survive and very few which showed half the lung surface consolidated.

TABLE 2.

Behaviour of four virus types in regard to inhibitors after heating to 56° C. for 30 minutes.

Type	Number	Origin	Meconium	Meconium/WSE	Sheep mucoid
MEL	85840	0	<2	<2	<2
WSE	85844	0	4·0	<2	4·5
M+	85847	0	3·7	3·0	<2
	86118	LD1	4·0	3·0	<2
	85782	0	3·7	3·0	<2
WS—	85845	0	<2	<2	3·5
	86069	LD1	<2	<2	3·5
	86355	LD2	<2	<2	3·5

0 = fluid from original limit titration of 1st cycle fluid.
LD1; LD2 = fluid from first or second limit titration of original fluid.

TABLE 3.

Behaviour of four virus types in virulence tests.

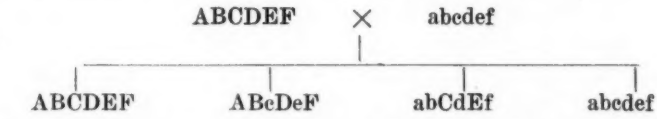
Type	Number	Origin	Chorioallantois	Mouse intranasal
MEL	85827	0	N N —	3 2 1
	85829	0	— — —	1·5 1 0
	85840	0	N —	2·5 1·2 0·5
WSE	85844	0	S S S	5 5 5
	85808	LD1	S S S	5 5 4
M+	85847	0	S S	1·0 1·5 2·3
	87189	0	N — —	0·5 0·5 1·8 2·0
	86916	2LD+1	S S S S	0·5 1·0 2·0 2·5
	86913	1LD+1	— — — —	0 0 1·0 1·5
WS—	85845	0	N N	5 5 5
	85848	0	— —	5 5 5
	86355	LD2	— — N	5 5 5 5

S = death of embryo with specific haemorrhages
N = death without haemorrhagic lesions
— = living embryo without lesions
0–5 = mouse lung lesions conventionally scored; 4 = death 6th or 7th day, 5 = death 3rd–5th day, 0–3·5 = degrees of consolidation of lung in surviving mice.

Symbols for origin as Table 2. 2LD+1: Two limit dilution passages followed by one subinoculation.

DISCUSSION.

These results are obviously similar to those obtained in recombination studies with bacterial viruses (Delbrück and Bailey, 1946; Hershey and Rotmann, 1949). Under conditions where one can expect to obtain the progeny from a large number of multiply-infected cells four types of virus can be obtained, the two original forms and two new types. The new types show some variation from strain to strain but typical examples show a symmetrical relationship which we have symbolized in relation to the originals as:



The groups of characters ABDF or abdf appear to be firmly linked and on the assumption that the serological character is the basic one we can regard these as non-transferable characters. The other two characters are well defined and regular in the parent strains but show more variability in the two new types. Nevertheless, the general picture is to find that the new types represent a simple transposition of these two transferable characters ABDF←CE→abdf.

Since the new types breed true we must be concerned with some sort of genetic change. For the reasons given in the preceding paper we can rule

out any suggestion that the new types arise by mutation or by any type of host environmental influence. They undoubtedly arise as a result of some form of genetic interaction and will henceforth be referred to as the products of that interaction.

The major challenge presented by these results is to derive from them information in regard to the mode of virus multiplication in the cell. The first implication is simply that they are inexplicable unless we postulate a particulate genetic mechanism. The transferable blocks of qualities must have genetic representation by at least 2 units with what must be regarded as allelomorphic equivalents in the two parent virus types. The second implication is that multiplication must be by some means other than simple binary fission. This of course merely confirms the very marked trend of current opinion on the point. The possible alternatives to binary fission need to be considered in the light of the probability that in the system we are considering a very considerable proportion of the haemagglutinating particles produced must be nonviable. For an H.A. titre of 160 fully infective virus should give a titre of about $10^{8.5}$ as against $10^{6.5}$ obtained with the one-cycle fluid. We may well be concerned only with the 1 p.c. of viable virus particles that are produced.

On the present results we can hardly avoid the conclusion that influenza viruses have at least as definite a genetic mechanism as bacteria and bacterial viruses, and that it conforms in at least a primitive sense to the universal pattern found in other living forms. We may provisionally ascribe each of the 6 phenotypic differences to differences between allelomorphic genes.

It will need much further work before the nature of the process by which segregation of characters occurs can be determined. The general form of the alternatives is similar to that presented by the phenomena of recombination with *E. coli* K.12 (Lederberg, 1947). (1) The genes may have a linear distribution on a single chromoneme and the results obtained are due to simple cross-over. (2) The genes may multiply independently and the genetic mechanism be reconstituted from the pool of genes so produced. Of the very large number of possible recombinants only the 4 types are viable. This is essentially the same as Luria's (1950) hypothesis of the nature of bacterial virus multiplication. (3) The phenomena may be of the same character as transformation reactions in the pneumococci. Certain genes readily escape from the genetic complex leaving a place which can be filled by a similar allelomorphic gene from another virus complex.

Work is continuing in an attempt to find data which might help to differentiate between these alternatives.

SUMMARY.

A method has been developed to obtain the product of first-cycle virus multiplication in cells of the allantoic cavity each infected with one or more virus particles of two different strains of influenza virus A.

Using the strains MEL and WSE which differ by 6 easily recognizable characters a first-cycle fluid has been studied which in addition to the parent forms contains two modified forms in which two sets of characters have been reciprocally transferred. The numbers isolated of the four forms are of the same order, 22 and 4 of the parent types to 9 and 6 of the new forms.

This is regarded as definitive evidence of genetic interaction between influenza viruses and a brief discussion of the possible mechanisms is given.

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THE PYROGENIC EFFECT OF THE INFLUENZA-MUMPS GROUP OF VIRUSES IN THE LABORATORY RABBIT

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The clinical symptoms of fever, muscle pain and headache typically accompanying infection with the influenza virus have prompted a number of workers to investigate this virus for its toxic properties. Henle and Henle (1946) found that large doses of influenza virus injected intra-cerebrally into mice produced tonic and clonic convulsions some 12-48 hours after inoculation. This effect was abolished by treating the virus with formalin, heat, ultra-violet light and by specific antiserum. Evans and Rickard (1945) produced a corneal opacity by injecting influenza A or B virus into the anterior chamber of the rabbit's eye. The virus did not multiply and the effect could be neutralized with specific antiserum.

Wagner, Bennett and Lequire (1949) investigated the response of rabbits to the intravenous injection of influenza viruses. They found that allantoic fluid containing influenza virus produced a sharp rise in temperature. All the evidence pointed to the virus itself being the pyrogenic agent. Specific immune serum neutralized the effect and it disappeared when the virus suspension had been heated to temperatures sufficient to destroy the haemagglutinating activity. In a second paper Bennett, Wagner and Lequire (1949) followed up this work and showed that rabbits tolerated a second injection of the homologous virus given on the second day. This tolerance was gradually lost and after about eleven days the rabbits were completely susceptible despite a high titre of circulating antibody. They also stated that the tolerance to second day injections of heterologous virus fell into a definite order corresponding to the position of the viruses in the receptor gradient of Burnet, McCrea and Stone (1946). They concluded that the pyrogenic effect was the result of union between the virus particle and a receptor substance. Wagner and Bennett in a third paper studied the effect of *V. cholerae* filtrate and *Cl. welchii* toxin upon the febrile reaction. They found that by giving near lethal doses of these substances on one day, the febrile response to an injection of influenza virus the next day was modified. This was considered to lend support to the hypothesis that union of virus and host receptor substance plays a part in the production of fever by these viruses.

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The production of high titre relatively pure *V. cholerae* receptor-destroying enzyme (Ada and French, 1950) and the study of its action in animals (French and Ada, 1950) in this Institute prompted us to attempt to confirm the work of Wagner *et al.* We have readily succeeded in confirming the pyrogenic effect of influenza virus and the tolerance of rabbits to a second injection of homologous virus. The results of our experiments with *V. cholerae* receptor-destroying enzyme do not, however, support the contention that enzymic destruction of receptor substances is the basis of the tolerance to repeated injections of virus.

Materials and Methods.

Animals. Adult rabbits of either sex bred in the Institute animal house were used throughout. These animals were of mixed breed and weighed about 2,000 to 3,500 gm. when used. The animals were brought to the experiment room the day previously and on the day of the experiment three rectal temperatures at 30-minute intervals were recorded and averaged for a base line. The virus preparations were injected slowly into the marginal ear vein and the rectal temperatures recorded in most experiments every 30 minutes. Unless mentioned in the text a new group of rabbits was used in each experiment.

Virus preparations. The classical influenza strains MEL (A), LEE (B), swine influenza virus and the Enders strain of mumps virus were used. These viruses were grown in the allantoic cavity of the developing chick embryo by the method of Burnet (see Beveridge and Burnet, 1946).

High titre allantoic fluids, free of red blood corpuscles and bacterial contamination, were stored until required in 2 ml. quantities sealed in glass ampoules in the dry-ice cabinet at —70° C. The titres of these fluids varied from 1: 512 to 1: 1024 as determined by a modified Salk test used routinely in this Institute (Anderson, Burnet and Stone, 1946). When necessary, the virus preparations were diluted in sterile physiological saline.

Avoidance of bacterial pyrogens. All glassware was sterilized by baking at 160° C. for 2 hours. Physiological salt solution and normal allantoic fluid were tested on several occasions and produced a maximum rise in temperature in the rabbits of 1° C. or less. This was no greater than the temperature rise of uninoculated rabbits held under the same conditions.

EXPERIMENTAL.

The pyrogenic effect of influenza virus was readily demonstrated on a number of occasions with different viruses. Normal saline or normal allantoic fluid was always without effect.

A typical response is recorded in Fig. 1 showing the average temperature curve of 6 rabbits receiving an intravenous injection of influenza virus strain MEL. There is a lag period of from one to one and a half hours after the injection and then the temperature rises steadily for the next 3 hours or more when the curve begins to flatten off and by the next morning the temperature has returned to normal.

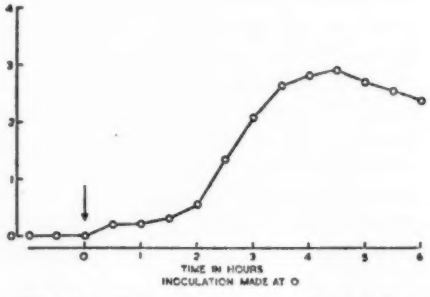

Fig. 1. Shows the average temperature curve in degrees Centigrade of 6 rabbits given 1·0 ml. of MEL virus intravenously.

The effect of a repeated injection of homologous or heterologous virus.

A series of eight rabbits were each given 1 ml. of MEL virus intravenously on day 1 and their temperature responses recorded. The following day two of these rabbits were again given 1 ml. of MEL virus and the temperature was again taken. Fig. 2 (a) shows the temperature fluctuations obtained on these

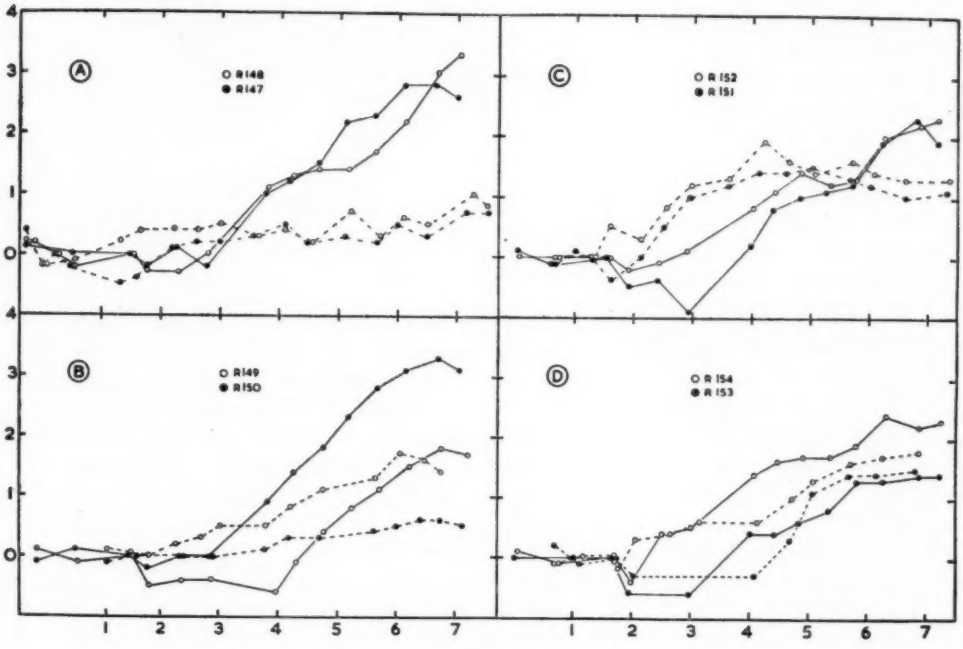

Fig. 2.

●——● } shows the temperature response to the initial injection of virus.
○——○ }

●·····● } shows the temperature response to the second injection of virus.
○·····○ }

(A) Second injection given 1 day after the initial injection.
(B) Second injection given 4 days after the initial injection.
(C) Second injection given 8 days after the initial injection.
(D) Second injection given 12 days after the initial injection.

two days. On each of days 4, 8 and 12, after the initial injection, two other rabbits from this series were taken and re-injected with 1 ml. of MEL virus. Figs. 2 (b), (c), and (d) show the temperature responses on these occasions. After 12 days the rabbits were again completely susceptible to the pyrogenic effect of MEL virus. To show that the resistance to a second injection was not

related to neutralization of the virus by antibody produced in response to the first injection, serum taken from the rabbits before each injection was titrated for anti-haemagglutinin. The results are shown in Table 1, and demonstrate that on day 2, when the rabbits are most resistant to second injections of virus, anti-haemagglutinin could not be detected.

TABLE 1.

Day	Rabbit	Preinjection	Postinjection
2	R 147	10	10
	R 148	10	10
4	R 149	10	30
	R 150	10	30
8	R 151	10	960
	R 152	10	960
12	R 153	10	320
	R 154	10	320

On the twelfth day when the anti-haemagglutinin titre is high the rabbits are almost fully susceptible to a second injection. The result is in agreement with and confirms the results of Bennett, Wagner and Lequire (1949).

To test the gradient hypothesis in connection with the pyrogenic effect of influenza virus the following experiment was made: Six rabbits each received 1 ml. of MEL virus and their temperature responses were recorded. The following day when all their temperatures had returned to normal the animals were re-injected, two with MEL, two with LEE and two with Swine virus. The haemagglutinin titres of these viruses were all adjusted to the same level. The temperature responses of the individual rabbits were again recorded. It was found that five of the six rabbits were partially tolerant to the second injection, the widest variation occurring in the two animals receiving homologous virus (MEL). This suggested that the first day injection had not completely rendered the animals tolerant to the second day injection. The following experiment was therefore made.

Two groups of rabbits were given 1 ml. of virus on the first day and on the second day 6 ml. of the same virus. One group of six rabbits was treated in this way with MEL virus and the other group with LEE virus. On the third day two rabbits from each group were challenged with 1 ml. of MEL virus, two with 1 ml. of LEE virus and two with 1 ml. of Swine virus. The maximum temperature responses on each day are shown in Fig. 3 A, B, C, D, E and F. These results indicate that large quantities of one virus given initially can render rabbits tolerant to other viruses given the next day. This result was further confirmed in the following experiment.

Six rabbits were given 3·5 ml. allantoic fluid containing mumps virus and two were given saline and their temperatures recorded. The following day the two rabbits that had received saline only were given mumps virus along with two other rabbits that had received mumps virus on the previous day. Two others like these latter received MEL virus and two LEE virus. The temperature responses on the two days are shown in Fig. 3 G and H. The initial dose of mumps virus can be seen to have left the rabbits tolerant not only to the homologous virus but also to the heterologous MEL and LEE viruses. The control rabbits that had received saline only on the first day were normally susceptible to the pyrogenic action of mumps virus on the second day.

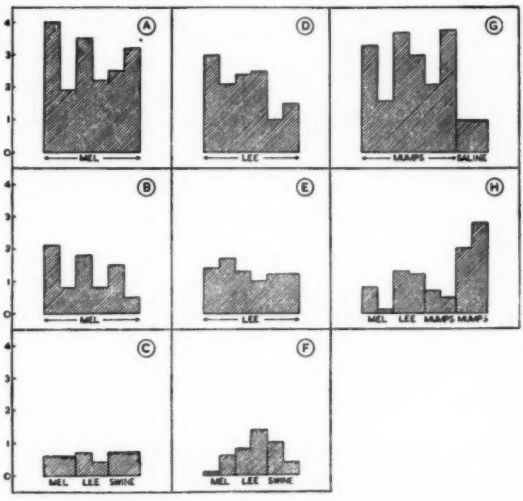

Fig. 3. The maximum rise in temperature above the base line is recorded for each rabbit.

3A temperature response of rabbits to 1 ml. of MEL virus given on day 1.

3B temperature response of same 6 rabbits to 6 ml. of MEL virus on day 2.

3C temperature response of rabbits to MEL, LEE and Swine viruses on day 3; two rabbits for each virus.

3D, E and F temperature response of rabbits similarly treated on days 1 and 2 with LEE virus and given MEL, LEE and Swine viruses on day 3.

3G temperature response of 6 rabbits given 3·5 ml. of mumps virus and of two rabbits receiving saline on day 1.

3H temperature response of rabbits receiving 1 ml. of MEL, LEE, or mumps viruses on day 2.

The effect of the receptor-destroying enzyme of V. cholerae *on the pyrogenic effect of influenza virus.*

Receptor-destroying enzyme of *V. cholerae* purified and concentrated by the method of Ada and French (1950) was given to rabbits in four daily intravenous injections over a period of two days. The total unitage given to each rabbit was $1{\cdot}5 \times 10^6$ units estimated by a technique slightly modified from Burnet and Stone (1947). This amount of RDE was contained in a total volume of 8 ml. Similar quantities of enzyme inactivated by heating at 56° C. for 30 minutes in the presence of citrate were given to other rabbits as controls

and two rabbits were given saline as a further control. Temperatures were taken on these rabbits but in every case remained normal.

On the third day each rabbit was given MEL virus and the temperature response recorded. Fig. 4 shows the average of 3 such experiments with 6 rabbits in each group. Despite the very large amounts of RDE given these rabbits were just as susceptible to the pyrogenic action of the virus as the rabbits given inactivated enzyme or saline.

Wagner and Bennett (1950) used simple broth culture filtrates of *V. cholerae* in the treatment of their rabbits. In an effort to simulate more closely the conditions used by these workers, crude *V. cholerae* culture filtrate containing 0·5 p.c. $CaCl_2$ and heated at 56° for 30 minutes (Burnet and Stone, 1947) was given six rabbits. Six other rabbits were given crude filtrate inactivated by heating in an excess of citrate at 56° C. for 30 minutes.

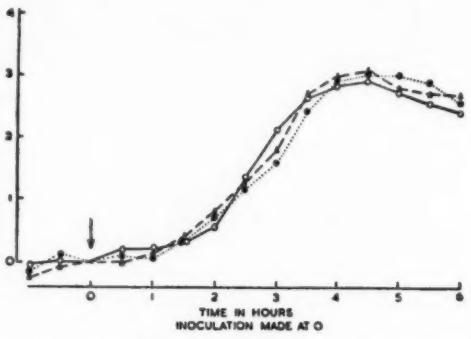

Fig. 4. Average temperature curves of 6 rabbits in each group receiving MEL virus after initial treatment with:

x.....x purified RDE
•.....• inactivated purified RDE
o———o saline.

A total of 10 ml. was given each rabbit in 4 injections over a period of 8 hours. The following day all but one of the rabbits receiving active and inactive crude RDE were dead. The one remaining rabbit that had received inactivated RDE was challenged with MEL virus. Three hours after the injection this rabbit exhibited a rise in temperature of only one degree (which can be considered to be within normal limits) and demonstrates the production of a resistance to the virus by the inactivated RDE. Due to the very toxic nature of the crude *V. cholerae* filtrates this experiment was not repeated.

The effect of heated non-enzymic virus on rabbits.

By heating influenza viruses under various conditions different results are obtained. Thus with LEE virus, heating at 56° C. for 30 minutes hardly affects the haemagglutinin titre of the virus but does destroy its power to act upon the red cell receptor or upon certain mucoid inhibitors of haemagglutination (Burnet, 1948). By raising the temperature to 70° C. the ability of virus to cause haemagglutination is destroyed.

Rabbits were given LEE virus unheated, heated at 56° C. for 30 minutes and heated at 70° C. for 30 minutes and their temperatures taken. Those rab-

bits receiving the unheated virus and virus heated at 56° C. for 30 minutes suffered a typical temperature response whilst the rabbits receiving virus heated to 70° C. for 30 minutes failed to react with a temperature. The next day all rabbits were challenged with active unheated LEE virus. The rabbits that had received the virus heated at 56° C. and unheated virus the previous day were unaffected by the second injection whilst the rabbit that had received previously the virus heated at 70° C. gave a typical temperature response.

Action of influenza virus on blood sugar level.

Delafield (1934) while studying the toxic and pyrogenic effect of antigenic fractions of *Salmonella typhimurium* on rabbits noted that these bacterial pyrogens produced first a hyperglycaemia followed later by a hypoglycaemia.

TABLE 2.

Blood sugar values in mg. per 100 ml.

Hours after injection of virus	R199 received active LEE virus	R200 received LEE virus heated at 56° C. for 30 min.	R201 received LEE virus heated at 70° C. for 30 min.
Before inoculation	100	100	143
1	110	106	113
3	120	103	115
5	88	—	118
24	105	—	—

Blood sugar determinations were made on the rabbits receiving active LEE virus, LEE virus heated to 56° C. for 30 minutes and LEE virus heated at 70° C. for 30 minutes. Table 2 shows the blood sugar values obtained in these rabbits and demonstrates that no significant change occurred in the blood sugar level. These results indicate that the mechanism involved in fever production by influenza viruses and by some bacterial pyrogens are different.

The in vivo *and* in vitro *neutralization of virus pyrogen by specific serum.*

We were a little puzzled by the findings in the experiment, the results of which are shown in Fig. 2, and Table 1, that although the sera of R153 and R154 had a considerable anti-haemagglutinin titre these rabbits were fully susceptible to virus pyrogen on the 12th day. This implied that antibody *in vivo* did not neutralize the pyrogenic effect of the virus and we therefore carried out the following experiments. Rabbits were given two injections of MEL virus at one week's interval and two weeks after the last injection of virus the level of the anti-haemagglutinin was determined. The rabbits were now bled and their serum used to neutralize *in vitro* a normally pyrogenic dose of virus and the

mixture inoculated into normal rabbits. The immunized rabbits also received a dose of virus. None of the animals suffered a temperature rise showing that the pyrogen had been fully neutralized both *in vitro* and *in vivo*. The original experiment in which only one injection of virus was given was therefore repeated with the following modifications. After twelve days the rabbits were bled and their serum used to neutralize virus. The virus-serum mixtures were inoculated into normal rabbits and virus was given to the rabbits donating the serum. All these rabbits reacted with a temperature rise which was somewhat delayed when compared with that of the normal control rabbit receiving virus alone. The anti-haemagglutinin titres of the rabbits receiving one and two injections respectively were comparable. It is possible that the antibody produced following one injection of virus is less avid than that produced following two injections.

DISCUSSION.

The results of the present investigation confirm the findings of Wagner *et al.* (1949) that intravenous influenza virus produces fever in rabbits. We have also confirmed the findings that rabbits are resistant to homologous virus given a day or so later. Wagner and his colleagues considered this latter phenomenon due to the enzymic action of the virus administered on the first day upon some receptor substance. In support of this idea they quoted their experiments with the receptor-destroying enzyme of *V. cholerae* (RDE) and with filtrates from cultures of *Cl. welchii*.

The production in this Institute of high titre non-toxic RDE (Ada and French, 1950) has enabled us to treat rabbits with much larger doses of enzyme than were used by Wagner *et al.* The 10 ml. of *V. cholerae* broth filtrate used by Wagner *et al.* would contain, at a generous estimate, not more than 20,000 units of RDE, as we have found simple broth cultures of *V. cholerae* to give a consistently low titre of enzyme. We gave our rabbits a total of $1{\cdot}5 \times 10^6$ units (at least 75 times more than Wagner *et al.*) in eight injections over a period of two days to allow full opportunity for enzymic action on receptors situated anywhere in the reticulo-endothelial system. The rabbits treated in this fashion were just as susceptible to the pyrogenic effect of influenza virus as control rabbits receiving equivalent amounts of inactivated enzyme.

Like Wagner and Bennett we found crude filtrates of *V. cholerae* cultures to be very toxic for rabbits. Out of six rabbits receiving 10 ml. of crude filtrate in 4 doses none survived 24 hours and only one out of six rabbits receiving crude filtrate *in which the RDE had been destroyed* by heat survived. It is significant that this rabbit failed to react with a temperature when given 1 ml. of MEL virus. This and the experience with purified RDE demonstrates that the protection afforded against the pyrogen of MEL virus by crude *V. cholerae* filtrates is not related to the RDE content.

In the present work we have been unable to demonstrate a gradient effect with the viruses studied. Indeed, the fact that when a relatively large amount of mumps virus [which is at the top (Burnet, McCrea and Stone, 1946) of the virus gradient suggested by Wagner and his co-workers] is given to rabbits they show tolerance to a wide range of viruses given on the second day, indicates that there is no virus gradient as far as the pyrogen phenomenon is concerned. This view is based upon the experiments in which large amounts of virus were given to the rabbits on the first day and the rabbits challenged the next day with homologous and heterologous viruses. No matter which virus was given on the first day, provided sufficient was given, the rabbits failed to show a temperature rise when given mumps, MEL, LEE or swine viruses the next day.

The experiments with heated LEE virus further demonstrate the non-essential nature of the virus enzyme in the pyrogen phenomenon. Wagner *et al.* found that heated virus, so long as it retained its capacity to haemagglutinate, would still produce a fever in rabbits. We have confirmed this finding. LEE virus heated at 56° C. for 30 minutes loses all enzymic activity as judged by its capacity to react with various mucoid substrates (Burnet, 1948), yet this non-enzymic LEE virus was able to render a rabbit resistant to a second injection of active virus the following day. LEE virus heated to 70° C. for 30 minutes neither produced a temperature in the rabbits nor did it protect them from active virus the following day.

It seems therefore (1) that the capacity to produce fever is a function of the virus particle, (2) that pyrogenic virus given in adequate amount to a rabbit renders a subsequent dose of virus ineffective as a pyrogen, (3) that active enzyme is not necessary for the pyrogenic action, and (4) that the host cells concerned are not modifiable by RDE. With the very large doses of RDE that were used we can feel certain that accessible receptors on any hypothetical fever-producing mechanism would be temporarily eliminated.

This suggests that the ionic configurations of the virus surface concerned with adsorption to red cells do not function as the pyrogen. It is probable, however, that the same essential units are concerned although their action in this instance is not mediated through an RDE sensitive receptor system. In this connection it is worth noting that when the haemagglutinin of an influenza virus preparation is destroyed by heat or strong urea solution the preparation also loses its serological reactivity (Burnet, 1951). The particular aspect of the virus surface involved in pyrogenic action might be the same as that responsible for its serological character but there is no positive evidence one way or the other.

SUMMARY.

Intravenous injection of a member of the influenza-mumps group of viruses in laboratory rabbits produces a rise in temperature in these animals beginning about 2 hours after the injection and reaching a maximum between the fourth and fifth hour.

Rabbits given large doses of virus in the form of infected allantoic fluid on one day, do not react with a temperature when given the same virus or another member of the group the following day. Virus heated sufficiently to destroy its enzymic capacity is effective as a pyrogen but when heated sufficiently to destroy its haemagglutinating capacity is no longer pyrogenic, nor can it protect the rabbit against a subsequent dose of pyrogenic virus. The blood sugar level of rabbits is not affected by the injection of virus pyrogen.

Purified receptor-destroying enzyme (RDE) of *V. cholerae* administered in large doses will not protect rabbits against the virus pyrogen.

It is concluded from these results:

(1) That the capacity to produce fever is a function of the virus particle,

(2) that pyrogenic virus given in adequate amount renders a subsequent dose of virus ineffective as a pyrogen,

(3) that active virus enzyme is not necessary for the pyrogenic action, and

(4) that the host cells concerned are not modifiable by RDE.

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RENAL REGULATION OF THE EXTRACELLULAR FLUID IN ACUTE RESPIRATORY ACIDAEMIA[1]

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The proposition has been advanced that when Na^+ is subtracted from the extracellular fluid in excess of Cl^- relative to the extracellular ratio of these two ions, the extracellular Cl/Na ratio remains constant. This is because the kidney excretes Cl^- in excess of Na^+ commensurately with the stress (Denton, Wynn, McDonald and Simon, 1951). If this statement is valid, the question arises, "What specific stimulus acts at the renal tubular level to cause the excess Cl^- excretion in excess Na^+ subtraction? i.e. how is the 'information' conveyed to the kidney tubules that twice as much Na^+ as Cl^- is being lost from the body through a duodenal fistula?" Two main possibilities were considered:

(a) That *the fall of extracellular pH* resulting from the excess Na^+ subtraction caused the excess Cl^- excretion.

(b) That the renal tubules *directly respond to the rise of extracellular Cl/Na ratio* caused by excess Na^+ subtraction. It appeared to us that significant information on this problem might come from comparison of renal behaviour in excess Na^+ subtraction (fall of extracellular pH—*direct* stress on extracellular Cl/Na ratio), with respiratory acidaemia (high alveolar carbon dioxide tension—fall of extracellular pH—no subtraction or addition stress directly distorting the relation of Cl^- to Na^+ in the extracellular fluid). The findings recorded in this paper suggest that when extracellular pH fall is caused by a relatively high alveolar carbon dioxide tension, the renal behaviour to electrolytes is not quantitatively similar to that seen when a comparable degree and duration of fall of extracellular pH is produced by excess Na^+ subtraction. However, as will be indicated in the discussion section, interpretation is not simple, since the raised alveolar carbon dioxide tension itself causes the relationship of Cl^- to Na^+ in the extracellular fluid to alter.

Experimental Method.

The technique used in experiments 6–14 was to place the animal's head in a small airtight cellophane bag; and the outlet of this was connected to a Coxeter Muschin anaesthetic machine. The CO_2 content of the circuit was controlled by the absorber of the Coxeter

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Muschin machine. The air in the circuit was cooled, and water vapour condensed by running the tubes through a bath of iced water. Frequent readings of the CO_2 and O_2 concentrations in the bag were made. O_2 was added to the circuit continuously, and the concentration was kept above 30 p.c. In experiment 10, a measurement of arterial O_2 was made, and was confirmed to be normal—19·8 ml. per 100 ml. In experiments 4 and 5 the sheep's head was placed in a large plastic bag approximately 2 feet cubed. Owing to the large dead space of the bag, it was very difficult to control its CO_2 content, and this method was replaced by the anaesthetic machine. It was found that increase of CO_2 tension in the circuit above 8 p.c. caused the animals to become very restless and distressed. Penicillin injections were given to the animals during the experiments. In R.A. 13 and 14, records of the animals' rectal temperature were kept and it was shown that it did not alter significantly. Experiments 1–3, the initial attempts to produce respiratory acidaemia, have not been included, as significant stress was not produced. Experiments 11–12 involved investigation of a separate problem, and have not been included.

Chemical Methods.

Most of these have been recorded previously (Harris, McDonald and Williams, 1952; Denton, McDonald, Munro and Williams, 1952). O_2 concentration was measured with a Beckman O_2 analyser. CO_2 concentration was measured with a Dwyer CO_2 analyser.

DESCRIPTION OF EXPERIMENTAL FINDINGS.

The following is a description of the main features of the findings in eight experiments in which severe respiratory acidaemia was produced in sheep.

This description covers the findings in R.A.'s 4, 5, 6, 8, 9, 10, 13 and 14. R.A. 7 represents a variation in the conditions of experiment, and will be referred to separately.

Alveolar carbon dioxide (the stress on ionic homeostasis).

It is clear from the analyses of the respired air in the respiratory circuit that the experimental technique succeeded in producing a high alveolar carbon dioxide tension for periods varying from 7 hours to 64 hours. The actual percentage of carbon dioxide in the circuit varied from two to over eight per cent. This is equivalent to a range of carbon dioxide tension from 15·2 to 60 mm. Hg. The rate and amplitude of respiration was in all cases raised. Simultaneous analyses showed that the oxygen content was kept at an average of 40-50 volumes p.c., the extreme range of variation being from 25-80 volumes p.c.

The following features of the experiments appear significant:

1. *Blood pH.*

This rise of CO_2 of the inspired air produced a large fall of blood pH (6·95–7·25). In R.A.'s 13 and 14 this fall was not as great as in the others, and possibly this was because the plasma HCO_3^- was high at the commencement of the experiment.

2. *Plasma bicarbonate.*

In all cases, there was a rise in the plasma bicarbonate concentration. In the light of the stress on ionic homeostasis, this may be regarded as a compensatory mechanism countering the stress on blood pH. The rise occurred rapidly in some instances, e.g. in R.A. 6, there was a rise of 14 m.eq. per litre in 5 hours. Table 1 shows that the P_{CO_2} of the blood, as calculated from the blood pH and total carbon dioxide content of plasma, was raised to the range of 70-120 mm. Hg. It is also indicated in Table 1 that if there had been this degree of rise of alveolar carbon dioxide with no rise of plasma bicarbonate above the original concentration the fall of blood pH would have been from 0·1 to 0·2 pH units greater than that found.

TABLE 1.

This table gives the values obtained for plasma total CO_2 content, pH and plasma CO_2 tension (P_{CO_2}) at various stages of the respiratory acidaemia experiments. pH* is the pH that would have resulted if the plasma HCO_3^- concentration had remained constant and the plasma CO_2 tension had altered as was found.

The calculations of P_{CO_2} were made from the nomogram of Singer and Hastings (1948).

Experiment	Time	Plasma HCO_3^- Concentration millimoles per litre	P_{CO_2} mm. Hg.	pH	pH*
Respiratory	22/8 1015	23·8	29·7	7·50	
	1530	40·4	105	7·17	6·92
Acidaemia	2345	40·6	92	7·23	6·98
	23/8 1010	43·0	112	7·17	6·89
No. 6	1600	42·0	101	7·20	6·94
	24/8 0035	42·4	101	7·21	6·94
	1150	36·6	96	7·16	6·96
	1615	34·5	58	7·37	7·18
Respiratory	9/10 1600	26·9	48	7·34	
	10/10 0800	33·6	76	7·22	7·13
Acidaemia	2200	31·1	58	7·32	7·25
	11/10 1000	34·3	78	7·23	7·12
No. 7	12/10 0000	34·5	84	7·19	7·08
	1000	34·8	65	7·31	7·20
	2100	31·5	62	7·29	7·22
Respiratory	20/11 1444	28·6	52	7·33	
	2300	33·2	118	7·02	6·94
Acidaemia	21/11 0900	39·9	>120	7·08	~6·92
	2100	40·7	99	7·20	7·03
No. 9	22/11 1300	44·2	>120	7·14	~6·92
	23/11 1000	35·8	50	7·47	7·34
	1830	35·0	39	7·56	7·45
Respiratory	11/12 1515	22·9	55	7·20	
Acidaemia	12/12 0900	32·1	116	7·04	6·86
	2200	33·7	111	7·05	6·88
No. 10	13/12 1030	34·8	92	7·15	6·96
	1425	35·9	70	7·29	7·09

> greater than ~ approximately.

3. *Relationship of Cl^- to Na^+ in the extracellular fluid.*

In these experiments the rise of bicarbonate ion in the extracellular pattern was accompanied by a fall in extracellular Cl/Na ratio. A gradual reduction of plasma chloride is a uniform finding in all experiments except No. 14, where only a very small decrease occurred. Generally it has taken 24-48 hours for a fall of 5-10 m.eq. per litre to be produced. The changes in sodium ion concentration are not as consistent. In R.A.'s 6 and 7 the initial rise in bicarbonate concentration was associated with a rise in sodium concentration relative to chloride. However, in the other experiments this was not the case, and it would appear that the fall in chloride is the overall consistent feature. The calculations below indicate that this change in extracellular Cl/Na ratio was not mainly a result of renal activity. It does not appear that the kidney consistently responds to a fall of extracellular pH caused by high alveolar carbon dioxide tension by a reduction of Na^+ excretion, a rising or constant Cl^- excretion, and hence the excretion of urine with a large amount of Cl^- in excess of Na^+.

Considering each experiment in detail:

Respiratory Acidaemia 4.

Duration of period of respiratory acidaemia, 7 hours.
Total excretion during this time, Na^+ 45·3 m.eq.
Cl^- 30·7 m.eq.
Cl/Na 0·68.

Thus, despite the marked fall in blood pH, the kidney excreted sodium in excess of chloride ion, and, in fact, the Cl/Na ratio of the urine fell relative to the pre-specimen.

Respiratory Acidaemia 5.

Duration of period of respiratory acidaemia, 13 hours.
Total excretion during this time, Na^+ 25·8 m.eq.
Cl^- 11·1 m.eq.
Cl/Na 0·43.

Thus, despite this sustained fall in blood pH, the kidney excreted sodium in excess of chloride in the urine. Relative to the initial finding, the urine Cl/Na ratio dropped during the course of the acidaemia.

Respiratory Acidaemia 6.

Duration of period of respiratory acidaemia, 51 hours.
Total excretion during this time, Na^+ 137·6 m.eq.
Cl^- 58·8 m.eq.
Cl/Na 0·43.

Here again it is shown that the kidney excreted sodium in excess of chloride despite the protracted fall of blood pH and the fall of extracellular Cl/Na ratio.

Sodium and chloride balance over period of respiratory acidaemia.

Initial plasma sodium concentration = 145 m.eq. per litre.
Initial plasma chloride concentration = 99·4 m.eq. per litre.
Initial plasma Cl/Na ratio = 0·67.
Total amount of sodium and chloride initially in the extracellular water[2].
Cl^- = 99·4 × 1·14 × 5·2 m.eq. = 588 m.eq.
Na^+ = 145 × 1·02 × 5·2 m.eq. = 774 m.eq.
Initial Cl/Na ratio in extracellular water, 0·76.
Total urine excretion: Cl^- 59 m.eq.
Na^+ 138 m.eq.

Therefore, predicted final Cl/Na ratio in E.C.W. $= \frac{588-59}{779-138} = \frac{529}{632} = 0{\cdot}84.$

Final Cl/Na ratio found in E.C.W. $= \frac{109}{149} = 0{\cdot}73.$

The extracellular water concentrations of Cl^- and Na^+ are calculated from the plasma values by the use of a Donnan factor of 0·95

$$Cl_{\text{E.C.W.}} = \frac{Cl_p}{W_p \times 0{\cdot}95} \text{ and } Na_{\text{E.C.W.}} = \frac{Na_p \times 0{\cdot}95}{W_p}$$

where W_p = gm. of water in 1 ml. of plasma—in most of these experiments assumed to equal 0·93 (Elkinton and Taffel, 1942).

Respiratory Acidaemia 9.

Duration of period of respiratory acidaemia, 63 hours.
Total excretion during this time, Na^+ 45 m.eq.
Cl^- 55 m.eq.
Cl/Na 1·22.

In this experiment, the sheep has excreted chloride in excess of sodium relative to extracellular Cl/Na ratio during the period of respiratory acidaemia. However, from the point of view of magnitudes, it can be noted that both the rate of excretion and the concentration of chloride in the urine have fallen progressively during the experiment. Moreover, relative to the pre-specimen Cl/Na ratio in urine, the ratio has with one exception been lower during most of the period of respiratory acidaemia.

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[2] In the sheep, it is not known what percentage of the body weight is extracellular fluid. Using the constant infusion technique, Schwartz, Schachter and Freinkel (1949) have shown inulin space in man to be 14 p.c. to 18 p.c. of body weight. In a study on a sheep with an almost empty rumen, the extracellular volume as measured by the constant infusion of inulin technique was 19 p.c. of body weight (Denton, McDonald, Williams and Munro, 1952). In that paper, the initial extracellular volumes of the other animals were calculated as 4 × the initial Congo Red space. In all instances except one (O.F.7–31 p.c.) the values were between 22–25 p.c. of body weight. If these values were too high, their effect on the calculations would result in an underestimate of the magnitude of Na^+ transfer. Estimations of the inulin space in sheep with both ureters tied, using a technique of single injection, ranged from 25–30 p.c. (Harris, McDonald and Williams, 1952). In these respiratory acidaemia experiments, the initial extracellular fluid volume has been assumed to be 20 p.c. of body weight. If the real values were higher than this, the renal contribution to the change of extracellular Cl/Na ratio was less than that indicated, and the validity of the main point of the calculation is unaffected. If the values were much lower it would follow that the kidney played a larger part in causing the pattern change than the calculations indicate. Such considerations do not affect the main issue, i.e. the renal excretion of electrolytes when there is a fall of pH in the extracellular fluid.

Sodium and chloride balance over period of respiratory acidaemia.

Initial plasma sodium concentration = 149 m.eq. per litre.
Initial plasma chloride concentration = 102 m.eq. per litre.
Initial plasma Cl/Na ratio = 0·69.
Total content of sodium and chloride initially in the extracellular water:

$$Cl^- = 102 \times 1{\cdot}11 \times 7{\cdot}7 = 872 \text{ m.eq.}$$
$$Na^+ = 149 \times 1{\cdot}02 \times 7{\cdot}7 = 1{,}147 \text{ m.eq}$$

Initial Cl/Na ratio in extracellular water = 0·76.
Total urine excretion: Cl^- = 51 m.eq.
Na^+ = 42 m.eq.

Therefore, predicted final Cl/Na ratio in E.C.W. $= \frac{872-51}{1147-42}$

$$= \frac{821}{1105} = 0{\cdot}74.$$

Final Cl/Na ratio found in E.C.W. $= \frac{102}{151} = 0{\cdot}67.$

Remarks. It would appear that the change in extracellular Cl/Na ratio is not mainly a result of renal activity. However, when the respiratory acidaemia stress ceased, the blood pH rose and the kidney excreted sodium in considerable excess of chloride. Whereas in the previous 24 hours the kidney had excreted 11·4 m.eq. of sodium and 11·1 m.eq. of chloride, in the 24 hours following respiratory acidaemia the kidney excreted 36·5 m.eq. of sodium and only 4·9 m.eq. of chloride. This renal activity has contributed to the rise in extracellular Cl/Na ratio which occurred during this 24-hour post-acidaemic phase.

Respiratory Acidaemia 10.

Duration of period of respiratory acidaemia, 46 hours.
Total excretion during this time, Na^+ = 14 m.eq.
Cl^- = 31 m.eq.
Cl/Na = 2·21.

Sodium and Chloride balance over period of respiratory acidaemia.

Initial plasma sodium concentration = 144 m.eq. per litre.
Initial plasma chloride concentration = 102 m.eq. per litre.
Cl/Na = 0·71.
Total content of sodium and chloride initially in the extracellular water:

$$Cl^- = 102 \times 1{\cdot}14 \times 6{\cdot}46 = 750 \text{ m.eq.}$$
$$Na^+ = 144 \times 1{\cdot}02 \times 6{\cdot}46 = 949 \text{ m.eq.}$$

Initial Cl/Na ratio in extracellular water = 0·79.
Total urine excretion: Cl^- = 31 m.eq.
Na^+ = 14 m.eq.

Therefore, predicted final Cl/Na ratio in E.C.W. $= \frac{750-31}{949-14}$

$$= \frac{719}{935} = 0{\cdot}78.$$

Final Cl/Na ratio found in E.C.W. = 0·74.

Remarks. During the period of respiratory acidaemia, the kidney has excreted chloride in excess of sodium. However, the excretion is not sufficiently great to account for the change of extracellular Cl/Na ratio observed. Again, the chloride excretion has dropped progressively during the course of the experiment. Also the urine Cl/Na ratio has fallen progressively relative to the Cl/Na ratio of the pre-specimens.

Respiratory Acidaemia 13.

The conditions of this experiment differed from the previous ones in that a small saline infusion (130 m.eq. of Na^+ and Cl^-—Cl: Na = 1: 1) was given after the animal had been acidaemic for 34 hours. Theoretically, *relative to the extracellular fluid*, this would tend to increase the stress on pH.

Duration of period of respiratory acidaemia, 49 hours.

Total urinary excretion during this time Na^+ = 42 m.eq.
Cl^- = 56 m.eq.
Cl/Na = 1·33

Sodium and chloride balance over period of respiratory acidaemia.

Initial plasma sodium concentration = 140 m.eq. per litre.
Initial plasma chloride concentration = 98 m.eq. per litre.
Initial plasma Cl/Na ratio = 0·70.

Total content of sodium and chloride initially in the extracellular water:

$$Cl^- = 98 \times 1{\cdot}14 \times 6{\cdot}9 = 773 \text{ m.eq.}$$
$$Na^+ = 140 \times 1{\cdot}02 \times 6{\cdot}9 = 986 \text{ m.eq.}$$

and initial Cl/Na ratio in the extracellular fluid = 0·78.

Taking into account Na^+ and Cl^- intake and urine excretion, predicted final Cl/Na ratio in the extracellular fluid would be

$$\text{Cl/Na} = \frac{773 + 130 - 56}{986 + 130 - 42} = \frac{847}{1074} = 0{\cdot}79.$$

$$\text{Cl/Na ratio finally found in E.C.W.} = \frac{101}{144} = 0{\cdot}70.$$

Remarks. During the period of respiratory acidaemia the kidney has excreted Cl^- in excess of Na^+. However, this would not account for the lowering of extracellular Cl/Na ratio observed. With the exception of one clearance period (8), the urine Cl/Na ratio has not shown any significant rise on the pre-acidaemic figure. The infusion of saline has had little effect on urine ion excretion. A fall of haematocrit followed the infusion, and it is possible that there was a movement of either Na^+ and water out of the intracellular fluid, or of Cl^- into the intracellular fluid. Reciprocal movements of both these ions may have occurred. The fall of urine Cl/Na ratio which occurred in the post-acidaemic phase was probably a factor in the rise in extracellular Cl/Na ratio which was observed to occur then.

Respiratory Acidaemia 14.

This experiment represents a variation of conditions and procedure. First, for an undetermined reason, the initial plasma Cl/Na ratio was low and the HCO_3^- was high. This to a considerable extent mitigated the effect of the high alveolar CO_2 on the blood pH. During the first 48 hours there was a 7 m.eq. per litre rise of plasma HCO_3^- and only a small fall of plasma Cl/Na ratio. The kidney excreted 22 m.eq. of Na^+ and 35 m.eq. of Cl^-. Second, after 48 hours of respiratory acidaemia, the animal was infused with saline (Cl: Na = 1: 1) so that the renal response to a stress tending to raise the extracellular Cl/Na ratio could be observed.

Duration of period of respiratory acidaemia, 62 hours.

Total excretion during this time = Na^+ 65 m.eq.
Cl^- 95 m.eq.
Cl/Na 1·46.

Total amount of Cl^- and Na^+ initially in the extracellular water:

$Cl^- = 104 \times 1{\cdot}14 \times 8 = 949$ m.eq.
$Na^+ = 151 \times 1{\cdot}02 \times 8 = 1{,}232$ m.eq.
and the Cl/Na ratio = 0·77.

Rise in extracellular Cl/Na ratio predicted to result from saline infusion:

$$Cl^-/Na^+ = \frac{949 + 190}{1232 + 190} = \frac{1139}{1422} = 0{\cdot}80.$$

Accounting for urine excretion

$$Cl/Na = \frac{1139 - 95}{1422 - 65} = \frac{1044}{1357} = 0{\cdot}77.$$

$$\text{Cl/Na ratio finally found in E.C.W.} - \frac{118}{151} = 0{\cdot}78.$$

During the 12 hours when saline (190 m.eq. of Na^+ and Cl^-) was being infused, the kidney excreted 44 m.eq. of Na^+ and 60 m.eq. of Cl^-. There was a 3 m.eq. per litre fall of plasma HCO_3^-, a small rise of plasma Cl/Na ratio, and a fall of plasma pH. Considered over the whole period of the experiment, the urine excretion of Cl^- in excess of Na^+ was adequate to prevent a rise of ratio as a result of saline infusion.

Remarks. The urine Cl/Na ratio rose during the first twelve hours of acidaemia, but then fell until saline infusion was given. Saline infusion caused an equal rise in rate of Na^+ and Cl^- excretion.

Respiratory Acidaemia 7.

This experiment differs from the others in two ways which may be significant: (a) One month previously this animal had been submitted to a severe respiratory acidaemia stress, i.e. R.A. 6; (b) Prior to commencement of respiratory acidaemia, the animal's rumen was washed out and 191 m.eq. of sodium and 60 m.eq. of chloride were removed. That is, the respiratory acidaemia stress was associated with a small excess sodium subtraction stress.

Duration of period of respiratory acidaemia, 74 hours.

Total urinary excretion during this time, Na^+ 29 m.eq.
Cl^- 104 m.eq.
Cl/Na 3·58.

Sodium and chloride balance over period of respiratory acidaemia.

Initial plasma sodium concentration = 149 m.eq. per litre.
Initial plasma chloride concentration = 110 m.eq. per litre.
Initial plasma Cl/Na = 0·74.

Total content of sodium and chloride initially in the extracellular water:

$$Cl = 110 \times 1{\cdot}14 \times 5{\cdot}2 = 651 \text{ m.eq.}$$
$$Na = 149 \times 1{\cdot}02 \times 5{\cdot}2 = 790 \text{ m.eq.}$$
Initial Cl/Na ratio in extracellular water = 0·82.

Taking into account urine excretion, predicted final Cl/Na ratio in

$$\text{E.C.W.} = \frac{651 - 104}{790 - 29} = \frac{547}{761} = 0{\cdot}72.$$

$$\text{Final Cl/Na ratio found in E.C.W.} = \frac{103}{140} = 0{\cdot}74.$$

Remarks. In this experiment, the kidney excreted chloride in excess of sodium. The amount of chloride excreted in excess of sodium was sufficient to account for the lowering of extracellular Cl/Na ratio observed.

In the six hours subsequent to ruminal washout, there was a small rise in urine Cl/Na ratio. During the acidaemic stress, the urine Cl/Na ratio rose further as did the urine concentration of chloride. A high urine concentration of chloride relative to sodium was maintained during the acidaemic phase but also continued in the post-acidaemic phase.

4. *Other plasma cations.*

No consistent change of plasma K^+ concentration occurred.

5. *Other plasma anions.*

A possible mode of stress compensation permitting extension of the bicarbonate fraction would be provided if there was a recession in the anions other than chloride in the plasma pattern. Only in one place in these experiments has there been a fall in plasma protein of one gm.—equivalent to approximately 2·40 m.eq. There was no suggestion that alteration in plasma phosphate was a significant factor.

However, an interesting fact emerges from the cation-anion differences. Total cation has been calculated by the constant addition of eight m.eq. (Ca^{++} and Mg^{++}) to the Na^+ and K^+ determinations. On the anion side, the protein equivalence has been calculated as gm. p.c. $\times$ .2·43 (Van Slyke factor) = m.eq. per litre of protein anion and this has been added to the Cl^-, PO_4 and HCO_3 determinations. Consideration of the difference between the total cation and this anion sum over the whole series of experiments appears to indicate fairly consistently that there was a decrease in this factor of unmeasured anions during the period of respiratory acidaemia stress. This unmeasured anion fraction includes sulphates, lactates, and volatile fatty acids. In R.A. 14 a small decrease of lactate concentration was observed during respiratory acidaemia. On the other hand, it may have been that a decrease in the cation equivalence of protein was involved (Brock, 1946). The titration curve of plasma protein is such that over the range of pH involved there could have been a reduction of cation equivalence of 2–4 m.eq. per litre (Clark, 1948). Qualitative alteration of protein which reduced its cation equivalence might also have been involved.

Other features in blood chemistry. Red cell analyses.

No change in red cell sodium, potassium, inorganic or acid-soluble phosphate (R.A. 6) is apparent. R.A. 9 suggests that there may have been a decrease in red cell chloride during the period of respiratory acidaemia stress. In R.A. 13, the eosinophile counts showed a 50 p.c. decrease during the acidaemic phase.

Findings in the Urine.

Urinary pH.

There was no marked consistent change in urine pH, such as is seen to occur in excess Na^+ subtraction. Presumably this is because there is no sudden reduction in Na^+ excretion as is seen to occur in this latter stress. There was sometimes a large rise of urine pH when the bag was removed (R.A.'s 9 and 14).

Excretion of Na^+ and Cl^-.

The behaviour of the kidney to Na^+ and Cl^- ions has not been quantitatively similar to that seen when pH fall is caused by excess Na^+ subtraction. There has been little evidence of these ions. The renal response during the first 24 hours has been equivocal. After 36 hours there has generally been a reduction in the rate of excretion of both ions, but with Na^+ rate and concentration reduced slightly more than Cl^-, so that the urine Cl/Na ratio has been greater than the extracellular Cl/Na ratio. The sudden rise of plasma pH which sometimes followed removal of the bag was associated with a rapid rise of urine Na^+ excretion and pH, but no change of Cl^- excretion.

In contrast to the situation in excess Na^+ subtraction, saline infusion has not resulted in differential excretion of these two electrolytes. In R.A. 14 there was an equal percentage increase in the rates of excretion after saline infusion.

In R.A. 7, in which respiratory acidaemia has been superimposed on a small excess Na^+ subtraction stress, the renal behaviour has been similar to that seen in excess Na^+ subtraction alone.

Bicarbonate ion.

In all experiments there was a large rise in plasma HCO_3^-. There was never any marked rise in urine HCO_3^- during the acidaemia. In no case did the kidney concentrate HCO_3^- ion in the urine relative to plasma concentration, even though in some cases plasma HCO_3^- concentration rose to almost double the pre-level. In the post-acidaemia phase, HCO_3^- excretion rose to as much as 10–25 times the rate during acidaemia, even though plasma concentration was falling.

In R.A. 6 there was an initial fall of urine HCO_3^- excretion even though over the 5-hour period during which this collection was made the plasma HCO_3^- rose 14 m.eq. per litre. Subsequently there was a rise of HCO_3^- ion excretion to as high as 29 m.eq. per litre, this being coincident with a rise of Na^+ excretion. This was the highest figure for urine HCO_3^- recorded during respiratory acidaemia in the series of experiments. Thereafter, urine HCO_3^- fell, and between the 37th and 51st hours the concentration and excretion rate were equivalent to or below the pre-period reading even though the plasma HCO_3^- was 39 m.eq. per litre at the 39th hour. In R.A. 7 there was little alteration in the rate of excretion of HCO_3^- during the experiment. The concentration never rose above 5 m.eq. per litre despite the rise of plasma HCO_3^-. Between the 29th and 47th hours the urine HCO_3^- concentration was equivalent to the pre-period even though plasma concentration was 30–32 m.eq. per litre. In R.A. 9 there was a very small rise in urine HCO_3^- ion concentration during the experiment. By the time the plasma concentration had risen to 40 m.eq. per

litre, the urine concentration had risen to 7 m.eq. per litre. The highest recording made (29 m.eq. per litre) was during the post-acidaemic phase. By this time, the plasma HCO_3^- level had fallen to that recorded in the pre-period. In R.A. 10 there was a fall of urine HCO_3^- concentration—the plasma HCO_3^- rose 10-14 m.eq. per litre. In R.A. 13 plasma HCO_3^- rose 10 m.eq. per litre, yet no rise of urine HCO_3^- concentration occurred. In the post-acidaemic phase urine HCO_3^- rose 8–20 times the concentration during the acidaemic period and this was associated with a fall of plasma HCO_3^-. In R.A. 14 the kidney was concentrating HCO_3^- in the urine in the pre-period—103 m.eq. per litre. The concentration fell steadily to 2 m.eq. per litre at the end of the acidaemic period even though plasma HCO_3^- concentration rose during the acidaemic period. At the end of the acidaemic period, the HCO_3^- concentration rose in the urine twenty-fifty times.

Ammonium ion.

In all experiments there was at some stage a rise in NH_4^+ ion concentration in the urine. In some cases (R.A.'s 7, 9 and 13) this rise was to over 100 m.eq. per litre. The excretion rate fell during the post-acidaemic phase (e.g. R.A.'s 7, 9 and 13). The NH_4^+ excretion during acidaemia was very labile, e.g. R.A. 9, where it suddenly fell to 13 m.eq. per litre. Coincident with this, it was observed that the blood pH had temporarily risen to 7·32. The question arises as to what was the equivalent amount of anion which accompanied this NH_4^+ ion in the urine. There was a rise in PO_4^{--} excretion in most cases but not of a degree sufficient to account for the change in NH_4^+. In R.A. 9, where large fluctuations of NH_4^+ were seen, there was no rise in SO_4^{--} ion excretion. No measurements of organic acid excretion were made except that in R.A. 6, when lactate ion excretion in urine was measured. No marked rise was observed but this does not exclude the possibility, as in this experiment there was not a large rise of NH_4^+ excretion. The terminal phase of R.A. 13 provided interesting data on the fluctuation of NH_4^+ excretion. A large rise of NH_4^+ excretion was followed by a fall, but the excretion rate rose again in the post-acidaemic phase and appeared in this instance to represent the cation equivalent to the rapid rise in HCO_3^- excretion.[3]

Phosphate ion and potassium ion.

It would appear generally true that there was an increase in phosphate excretion during acidaemia. In most cases the urine concentration and excre-

[3] This large NH_4^+ excretion in an alkaline urine appeared to be unusual. We repeated all readings in duplicate, and the method of estimation was checked against standard solutions.

tion rate of potassium ion fell during acidaemia. In R.A.'s 9, 13 and 14 there was a rise in K^+ concentration in the post-acidaemia phase coincident with the rise in pH.

Glomerular Filtration Rate and Electrolyte Clearances.

There was no evidence of a fall of glomerular filtration rate occurring during respiratory acidaemia such as occurs in excess Na^+ subtraction. In some instances there was a clear cut rise during the course of acidaemia (R.A.'s 9, 10 and 14). There was sometimes a fall of glomerular filtration rate during the post-acidaemic phase (R.A.'s 9, 13 and 14).

This finding, plus the fact that HCO_3^- ion concentration in the plasma rose 20-50 p.c., indicates that urine HCO_3^- excretion has remained low even though there has been substantial increase in the filtered load of HCO_3^- ion. This is well shown in the series of clearances done on R.A. 13. Even though one of the pre-acidaemic glomerular filtration rates was higher than any during the acidaemic phase, the HCO_3^- load has been consistently higher during the latter part of the acidaemic phase (Clearances 6, 7, 8, 9, 10), and yet the HCO_3^- ion excretion rate has been 1/3–1/12 as large. In R.A. 10 no rise of HCO_3^- ion excretion rate has occurred even though G.F.R. determinations and plasma concentration rise indicate that the HCO_3^- load has doubled. In R.A. 14 the HCO_3^- load has risen during acidaemia yet the excretion rate has fallen.

During the post-acidaemic phase, the plasma HCO_3^- ion concentration has fallen, and in most instances the G.F.R. has fallen. In R.A. 13, despite decrease of HCO_3^- load from 4/5–1/3 of that seen in the latter phase of acidaemia, the excretion rate was 2-12 times greater than during the acidaemic phase. In R.A. 14 there was a thirty-five-fold rise in the rate of HCO_3^- ion excretion during the post-acidaemic phase even though there was a 25 p.c. decrease of HCO_3^- load.

The decline of Cl^- ion load which would have occurred as a result of plasma concentration decrease was countered in some instances by rise of G.F.R. (R.A.'s 9 and 10). Nevertheless, the rate of Cl^- ion excretion fell. In R.A. 13, with one exception (Cl. 7), the excretion rates during acidaemia were lower than the pre-acidaemic. The load in Cl. 6 was higher than in Cl. 2 of the pre-acidaemic period, yet the excretion rate was one-half as great. Chloride ion excretion fell in the post-acidaemic period. There was sometimes a sharp rise of Na^+ and K^+ excretion rates at this time even though the filtered loads were falling.

DISCUSSION.

The experimental findings made do not permit of a definite statement of the renal behaviour in acute respiratory acidaemia in the sheep. It would seem probable, however, that a negative conclusion on the main problem is legitimate. That is, the renal behaviour to the main extracellular electrolytes

CLEARANCE STUDIES—R.A. 13.

Time and Clearance No.	ml. H_2O filtered per min.	ml. H_2O reabsorbed per min.	ml. H_2O excreted per min.	p.c. reabsorbed	p.c. excreted	Electrolyte	m.eq. filtered per min.	m.eq. reabsorbed per min.	m.eq. excreted per min.	p.c. reabsorbed	p.c. excreted
Pre-acidaemia Clearance (1) —20 hours	93·8	89·3	4·5	95·2	4·8	Na^+	13·132	12·946	0·186	98·59	1·41
						Cl^-	9·286	9·109	0·177	98·39	1·61
						K^+	0·422	0·281	0·141	66·59	33·41
						HCO_3^-	2·899	2·817	0·072	97·52	2·48
Clearance (2) —19 hours	76·8	75·2	1·6	97·9	2·1	Na^+	10·752	10·704	0·048	99·56	0·44
						Cl^-	7·526	7·440	0·076	99·0	1·0
						K^+	0·330	0·271	0·059	82·11	17·89
						HCO_3^-	2·334	2·320	0·014	99·40	0·60
Clearance (3) 10 hours	64·4	60·3	4·1	93·6	6·4	Na^+	9·144	9·128	0·016	99·83	0·17
						Cl^-	6·311	6·286	0·025	99·61	0·39
						K^+	0·238	0·214	0·024	89·92	10·08
						HCO_3^-	2·318	2·302	0·016	99·32	0·68
Clearance (4) 12 hours	73·9	72·8	1·1	98·6	1·4	Na^+	10·493	10·481	0·012	99·89	0·11
						Cl^-	7·168	7·148	0·020	99·73	0·27
						K^+	0·221	0·203	0·018	91·96	8·14
						HCO_3^-	2·660	2·657	0·003	99·89	0·11
Clearance (5) 22 hours	74·1	65·5	8·6	88·4	11·6	Na^+	10·441	10·390	0·051	99·51	0·49
						Cl^-	7·330	7·262	0·068	99·08	0·92
						K^+	0·259	0·216	0·043	83·21	16·79
						HCO_3^-	2·789	2·764	0·017	99·39	0·61
Clearance (6) 23 hours	86·6	84·6	2·0	97·7	2·3	Na^+	12·210	12·182	0·028	99·86	0·14
						Cl^-	8·227	8·189	0·038	99·59	0·41
						K^+	0·363	0·335	0·028	92·32	7·68
						HCO_3^-	3·410	3·404	0·006	99·83	0·17

CLEARANCE STUDIES (Contd.)—R.A. 13

Time and Clearance No.	ml. H_2O filtered per min.	ml. H_2O reabsorbed per min.	ml. H_2O excreted per min.	p.c. reabsorbed	p.c. excreted	Electrolyte	m.eq. filtered per min.	m.eq. reabsorbed per min.	m.eq. excreted per min.	p.c. reabsorbed	p.c. excreted
Clearance (7) 30 hours	78·9	76·4	2·5	96·8	3·2	Na^+	11·046	10·974	0·072	99·35	0·65
						Cl^-	7·337	7·255	0·082	98·89	1·11
						K^+	0·331	0·294	0·035	89·45	10·55
						HCO_3^-	3·124	3·109	0·015	99·52	0·48
Clearance (8) 34 hours	73·5	71·9	1·6	97·9	2·1	Na^+	10·290	10·276	0·014	99·87	0·13
						Cl^-	6·615	6·571	0·044	99·34	0·66
						K^+	0·264	0·244	0·020	92·64	7·56
						HCO_3^-	2·969	2·953	0·016	99·47	0·53
Clearance (9) 37 hours	74·5	72·9	1·6	97·8	2·2	Na^+	9·685	0·669	0·016	99·84	0·16
						Cl^-	6·407	6·383	0·024	99·63	0·37
						K^+	0·238	0·217	0·021	91·18	8·82
						HCO_3^-	2·920	2·898	0·022	99·27	0·73
Clearance (10) 47 hours	77·8	73·3	4·5	94·1	5·9	Na^+	11·125	11·089	0·036	99·68	0·32
						Cl^-	7·002	6·939	0·063	99·11	0·89
						K^+	0·241	0·219	0·022	90·88	9·12
						HCO_3^-	3·034	3·013	0·021	99·34	0·66
Post-acidaemia Clearance (11) 51 hours	49·2	48·4	0·8	98·4	1·6	Na^+	6·740	6·719	0·021	99·69	0·31
						Cl^-	4·280	4·274	0·006	99·84	0·16
						K^+	0·196	0·155	0·041	79·09	20·91
						HCO_3^-	1·918	1·823	0·085	95·6	4·4
Clearance (12) 54 hours	73·2	71·9	1·3	98·3	1·7	Na^+	7·955	7·945	0·010	99·88	0·12
						Cl^-	6·441	6·422	0·019	99·71	0·29
						K^+	0·285	0·246	0·039	89·81	10·19
						HCO_3^-	2·488	2·428	0·060	97·6	2·4
Clearance (13) 72 hours	40·0	33·0	7·0	82·5	17·5	Na^+	5·080	5·045	0·035	99·31	0·69
						Cl^-	3·160	3·132	0·028	99·11	0·89
						K^+	0·132	0·076	0·056	57·58	42·42
						HCO_3^-	1·328	1·284	0·044	96·99	3·01

CLEARANCE STUDIES—R.A. 14.

Clearance No.	G.F.R.	ml. water reabsorbed per min.	ml. excreted per min.	p.c. reabsorbed	p.c. excreted
2 2318–2328 21st	87.0	82.70	4.30	95.06	4.94
3 2245–2305 22nd	101.0	98.1	2.90	97.13	2.87
4 0131–0151 23rd	76.0	75.10	0.90	98.82	1.18
5 1638–1648 23rd	56.40	54.40	2.00	96.45	3.55

Electrolyte	m.eq. filtered per min.	m.eq. reabsorbed per min.	m.eq. excreted per min.	p.c. reabsorbed	p.c. excreted
Na^+	13.137	13.117	0.020	99.85	0.15
K^+	0.287	0.261	0.026	90.95	9.05
Cl^-	8.874	8.851	0.023	99.74	0.26
HCO_3^-	3.219	3.204	0.015	99.53	0.47
Na^+	15.150	15.005	0.145	99.04	0.96
K^+	0.354	0.351	0.003	99.16	0.84
Cl^-	10.504	10.298	0.206	98.14	1.96
HCO_3^-	3.595	3.589	0.006	99.84	0.16
Na^+	11.248	11.075	0.173	98.46	1.54
K^+	0.243	0.232	0.011	95.47	4.53
Cl^-	7.904	7.682	0.222	97.19	2.81
HCO_3^-	2.710	2.621	0.089	96.70	3.30
Na^+	8.234	8.070	0.164	98.00	2.00
K^+	0.192	0.098	0.094	50.50	49.50
Cl^-	5.809	5.785	0.024	99.59	0.41
HCO_3^-	1.677	1.674	0.003	99.82	0.18

is not quantitatively similar to that seen when extracellular pH fall results from excess Na^+ subtraction. In excess Na^+ subtraction, it is often seen that renal excretion of Na^+ is 1-30 m.eq. per litre, and Cl^- is 40-120 m.eq. per litre.

With regard to the main problem stated in the introduction, there would seem to be two possibilities:

(a) Whilst in both situations there is a comparable pH fall, in excess Na^+ subtraction there is the added factor of direct subtraction distortion of the extracellular Cl/Na ratio, and it is this additional factor which determines the difference between the two situations.

(b) That the renal response of Na^+ conservation and large Cl^- excretion which might be a commensurate effect of plasma pH fall has been prevented by there being at least one new factor in the situation of respiratory acidaemia: that is from the formal viewpoint of comparison with excess Na^+ subtraction, interpretation would be simpler if respiratory acidaemia did not itself cause the extracellular Cl/Na ratio to alter.

It can be stated that the evidence available for comparison with this paper (Denton, Wynn, McDonald and Simon, 1951; Denton, McDonald, Munro and Williams. 1952) does not permit a conclusion. However, examination of the coincident factors suggests that (a) is more probable.

1. In respiratory acidaemia, there is little alteration of Cl^- load.[4] In excess Na^+ subtraction there is a large fall (Denton, McDonald, Munro and Williams, 1952), yet Cl^- excretion is greater. If it were argued that the amount of electrolyte excreted by the kidney is influenced by the load of electrolyte presenting to the renal tubules, it would appear that some factor operates in excess Na^+ subtraction which does not act in respiratory acidaemia.

2. Non-specific trauma in sheep causes an alteration in the extracellular electrolyte pattern. There is a fall in the Cl/Na ratio and a rise in the HCO_3 ion. It has been a uniform finding in these circumstances that the kidney excretes Na^+ in excess of Cl^- (Denton, Wynn, McDonald and Simon, 1951; Harris, McDonald and Williams, 1952; Denton, McDonald, Munro and Williams, 1952). In relation to magnitudes, the following facts seem relevant. In the instance of the uncomplicated abdominal operation, the plasma pattern change is not an invariable occurrence. When it occurs the effect is not large—3–7 m.eq. per litre rise in HCO_3^-, the maximum effect in our studies being a rise of 5 m.eq. per litre in HCO_3^- persisting 48 hours after operation. However, when the operation is that of ureteral ligation, the effect appears to be enhanced, and rises of as much as 13 m.eq. per litre in plasma HCO_3^- are

[4] This finding is not inconsistent with the fact that increase of blood CO_2 tension increases cardiac output (Jerusalem and Starling, 1910). There may also be a high cardiac output in pulmonary emphysema, but the determining factors are probably different (Harvey, Ferrer, Richards and Cournand, 1951).

recorded (Harris, McDonald and Williams, 1952). The operation of oesophageal fistula causes this non-specific trauma change, but the effect it has on urine excretion does not persist longer than 24-36 hours. The factors which determine electrolyte excretion in excess Na^+ subtraction predominate quantitatively by this time.

There is no direct evidence in these respiratory acidaemia experiments that this non-specific pattern change occurred. There was no operative performance on the animals within 20 hours of commencement of the acidaemia, and, as indicated, it has been observed that even laparotomy under general anaesthesia may not initiate this pattern change. However, this important objection cannot be positively excluded, particularly in the first 24 hours when urine ion excretion was equivocal. The findings in R.A. 6, where Na^+ was excreted in excess of Cl^-, are particularly suggestive, but even here the rapid post-operative rise of HCO_3^- was associated with Cl^- in excess of Na^+ in the urine. In those experiments lasting more than 24 hours, there was not a large renal response to acidaemia, but it was a uniform finding that the urine contained a small amount of Cl^- in excess of Na^+ (R.A.'s 9, 10, 13, 14 and 7—exception R.A. 6). This is contrary to the observed renal behaviour in the non-specific trauma reaction. Moreover, the rapid renal response with rise of Na^+ excretion which occurred after removal of the bag is suggestive that the renal behaviour prior to this was determined by factors consequent on the high alveolar CO_2. It can be noted also that the dimension of pattern change in respiratory acidaemia (HCO_3^- increase of 10-17 m.eq. per litre) is greater than that of uncomplicated non-specific trauma reaction. Finally, it can be added that in the operation for excess Na^+ subtraction the change did occur and was quantitatively overshadowed in 24-36 hours.

The finding that the urine Cl/Na ratio was greater than extracellular Cl/Na ratio even though the latter was decreased is interesting, and is similar to the urine findings in chronic respiratory acidaemia in man. In excess Cl^- subtraction (fall of extracellular Cl/Na ratio—rise of extracellular pH) the urine contains Na^+ in excess of Cl^- (Denton, Wynn, McDonald and Simon, 1951). At the other extreme, in excess Na^+ subtraction (rise of extracellular Cl/Na ratio—fall of extracellular pH) the urine contains Cl^- in great excess of Na^+. The urine findings in the experiments recorded here and in chronic respiratory acidaemia in man (fall of extracellular Cl/Na ratio—fall of extracellular pH), appear to represent a position intermediate between the two extremes. In R.A. 7 the small added excess Na^+ subtraction stress appeared to potentiate the renal response to pH fall. Comparison of the data here with excess Na^+ subtraction suggests that some factor other than pH has direct influence on renal behaviour, but it does not follow that pH change has no influence. It is suggestive that pH change is a factor in the difference of renal response in excess Cl^- subtraction and respiratory acidaemia. Thus the cor-

rect answer to the question of which factor acts at the renal level in excess Na^+ subtraction may be that both do, the end result being a synergistic effect, and the problem depends on quantitatively defining their rôles.

Bicarbonate ion.

The data presented here do not support a proposition that the renal excretion of HCO_3^- ion is determined by whether or not, and by how much, the filtered load exceeds the functional reserve of a tubular system responsible for active transport of HCO_3^-. There has often been little HCO_3^- ion excretion when filtered load was maximal, and a large excretion when filtered load has been reduced, the significant difference in the two situations appearing to be change in the stress on the extracellular electrolyte pattern. These findings are consistent with the clinical observations that patients with emphysema have very little HCO_3^- ion in the urine even though plasma concentration is increased (Table 2.)

Clinical implications.

Because of the quite different time scale, these experiments give no information on the mechanism of pattern change in chronic respiratory acidaemia (pulmonary emphysema) in man. In the chronic *equilibrium* state of respiratory acidaemia in man, where there is a constant ionic intake of varying proportions, maintenance of the altered extracellular electrolyte pattern presumably would mainly depend on renal activity. Over a period of months no other mechanism could account for it. A four-day period of observation on the patient J.F. (Table 2) showed that the extracellular Cl/Na ratio remained constant at the lowered level, even though a large amount of Cl^- in excess of Na^+ was administered. There was a large Cl^- in excess of Na^+ excretion in the urine during this time. In a previous paper (Denton, Wynn, McDonald and Simon, 1951) we raised the possibility of K^+ depletion, resulting from acidaemia, being involved in the pattern change in respiratory acidaemia. There was no evidence of it in these experiments, and in the chronic state in man it seems likely that K^+ intake from food would be adequate to combat any increased loss resulting from acidaemia.

Consideration of the physiological consequence of the altered extracellular electrolyte pattern emphasizes the importance of normal renal electrolyte regulation in a patient with impaired pulmonary gas exchange. Such a patient with renal damage would appear to require especially careful biochemical care. Management of diet presumably should be such as to avoid a large Cl^- in excess of Na^+ intake. In this regard, the importance of certain future investigations is clear, e.g. what is the effect of congestive cardiac failure on the capacity of the kidney to maintain this altered pattern? Would a rise in plasma Cl/Na ratio and increasing acidaemia result? It would seem likely that excess Na^+ subtraction (e.g. diarrhoea) in a patient with respiratory acidaemia is a very serious complication.

TABLE 2.

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Patient	WHOLE BLOOD		CELLS			PLASMA							URINE					
	pH	Ht.	Na	K	Cl	Protein gm. p.c.	Na	K	Cl	HCO_3	pCO_2 mm. Hg.	$\frac{Cl}{Na}$	pH	Na	K	Cl	HCO_3	$\frac{Cl}{Na}$
			m.eq. per litre				m.eq. per litre							m.eq. per litre				
1 F.W.	7.30	43.6	17	94	58	7.08	145	3.6	103	28.4	59	0.71	5*	127	28.2	116		0.91
2 A.B.	7.39	49.2	14.8	97	56	6.85	142	4.1	98	28.6	49	0.69	5*	87	19.4	96		1.10
3 J.K.	7.42	52.5	14.0	96	56	7.16	144	3.3	98	28.5	45	0.68	5*	136	59	134		0.99
4 W.F.	7.32	50.0	14.2	100	57	6.85	144	4.2	98	33.3	67	0.68	5*	88	67	103		1.17
5 A.C.	7.30	49.0	14.4	98	58	6.41	145	3.5	96	35.5	73	0.66	7*	108	28.0	84		0.78
6 M.B.	7.35	46.0	14.0	91		6.77	156	3.7	102	28.9	53	0.65	5.35	148	54	137	0.44	0.93
7 J.F.	7.38	35.5	22	93		7.33	150	3.8	101	33.3	58	0.67	5.40	200	32	172	0.31	0.86
8 A.M.	7.43	46.5	15	90		9.48	145	3.9	95	32.4	51	0.66	5.4	176	48	169	0.64	0.96
9 A.H.	7.33	46.0	17	86		6.77	152	3.7	101	27.8	53	0.66	5.9	107	20	54	1.46	0.50

*** pH determination by Polychrome indicator.**

Plasma, red cell and urine analyses of a series of patients with emphysema. The characteristic extracellular pattern with low Cl/Na ratio, raised HCO_3^-, and lowered Cl^- is shown. The raised extracellular HCO_3^- has, in most cases, countered the effect of the raised alveolar carbon dioxide tension (pCO_2) on blood pH. The urine HCO_3^- excretion is very low, even though extracellular HCO_3^- level is raised. In most patients the urine contains Cl^- in excess of Na^+ even though the extracellular Cl/Na ratio is lowered. The haematocrit determinations are high in almost all cases. Cell values are given as m.eq. per litre of cells, not as m.eq. per litre of cell water.

SUMMARY.

A high alveolar CO_2 tension in sheep for 24-72 hours produced a large fall of blood pH.

A fall of plasma Cl/Na ratio, and a rise of plasma HCO_3^- was produced. The rise of plasma HCO_3^- to some extent countered the effect on extracellular pH of the high alveolar CO_2 tension (Table 1).

The renal response to this fall of blood pH was not quantitatively similar to that seen in excess Na^+ subtraction in sheep (Denton, McDonald, Munro and Williams, 1952). There was not a large fall of Na^+ excretion, a rising or constant Cl^- excretion, and hence the excretion of a urine of high Cl/Na ratio. The renal response was also different from that seen in excess Cl^- subtraction (extracellular Cl/Na ratio fall—rise of extracellular pH).

This evidence, when considered in conjunction with the evidence on electrolyte subtraction suggests that pH may directly modify renal electrolyte excretion. However, this does not invalidate the hypothesis that in excess Na^+ subtraction the rise of Cl/Na ratio of the glomerular filtrate influences renal tubular behaviour.

Despite the fact that both glomerular filtration rate and plasma concentration of HCO_3^- may rise, there was no increase of the rate of HCO_3^- excretion in the urine. When the stress of high alveolar CO_2 was discontinued, there was often a fall of glomerular filtration rate, and a fall of plasma HCO_3^-, and yet a sudden rise of HCO_3^- excretion.

Acknowledgments. We wish to thank Professor R. D. Wright and Dr. Geoffrey Kaye for their most helpful suggestions on experimental procedure, and Mr. E. Hughes for valuable technical assistance. We would also like to thank Dr. Lionel Bull and Dr. A. W. Turner, of C.S.I.R.O. Animal Health Division, and Mr. G. S. Coleman, of the Australian Estates Co., for the financial assistance which they generously provided.

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RESPIRATORY ACIDAEMIA. No. 4—8/8/50.

Time	Co_2 vols.	O_2 p.c.	Resp. rate per min.	Blood pH	Vol. ml.	Urine pH	Urine m.eq. per litre Na^+	Urine m.eq. per litre K^+	Urine m.eq. per litre Cl^-	Urine m.eq. per litre Cl/Na	Total excretion m.eq. Na^+	Total excretion m.eq. K^+	Total excretion m.eq. Cl^-	Urine ml. min.	Rate of excretion m.eq. per hour Na^+	Rate of excretion m.eq. per hour K^+	Rate of excretion m.eq. per hour Cl^-
1000				7·39	18	7·9	208	108	175	0·84	3·7	1·9	3·1				
1030				7·39													
1050	2·1		152														
1120	4·8	62	74	7·20													
1220	8	73															
1238	8·5	54		7·07	31	6·9	292	137	158	0·54	9·0	4·2	4·9	0·28	4·95	2·32	2·68
1255	4·2	32	42														
1305		28		7·13													
1330	6·2		78														
1430	5·6	45	68	7·20	39	7·9	277	92	184	0·66	10·8	3·5	7·1	0·35	5·90	1·96	3·92
1455				7·25													
1500			68	7·25													
1555	7·6	56	70			8·0											
1600	6·4	58															
1630	8·5	44	78	7·03													
1652		46	42														
1700			40		87	6·8	250	90	178	0·71	21·7	7·8	15·4	0·56	8·43	3·03	6.00
1707	4·8																

RESPIRATORY ACIDAEMIA, No. 5.

Time Hrs.	CO_2 vols. p.c.	O_2 vols. p.c.	Resp. rate per min.	Blood pH	URINE Vol. ml.	pH	Na^+	K^+	Cl^-	$HPO_4^{=}$	Cl/Na	TOTAL EXCRETION Na^+	K^+	Cl^-	HPO_4^-	RATE OF EXCRETION Urine ml./min.	Na^+	K^+	Cl^-	HPO_4^-
					m.eq. per litre							m.eq.				m.eq. per hour				
1005			140	7.38	128	7.19	22	14	22.6	1.3	1.03									
1100						7.95														
15	2.7	25	84																	
30				7.13																
1303				7.06																
15	2.7	31	92		45		110	28	82	12	0.74	5.0	1.3	3.7	0.54	0.23	1.7	0.45	1.2	0.17
45	6.6	25	90			6.70														
1400	7.0		82	7.09																
1530	5.1	56	48	7.28	37	6.74	138	53	52	17	0.38	5.1	2.0	1.9	0.63	0.25	2.1	0.82	0.8	0.26
1630	8+	57	94	7.19		6.38														
1730	7.4	76	68	7.13	27		171	56	53	17	0.31	4.6	1.5	1.4	0.46	0.25	2.6	0.83	0.8	0.26
40						6.79														
1910			102	7.08	28		165	60	75	17	0.45	4.6	1.7	2.1	0.48	0.25	2.5	0.93	1.1	0.26
45	4.7	40	38			6.39														
2045	2.8	34	56	7.4	17	7.0	157	88	50	15	0.32	2.7	1.5	0.9	0.27	0.17	2.5	1.4	0.8	0.25
2130		42	80			7.2														
50				7.23																
2225	8+	44	108	7.12																
50	8+			7.17	26		148	63	54	17	0.37	3.8	1.6	1.4	0.46	0.24	2.1	0.76	0.8	0.25
53				7.26																
2305				7.32																
Overnight							73	45	40	17	0.55									

* Phosphate in millimols

THE EFFECT OF ANOXIA ON PULMONARY OEDEMA PRODUCED BY MASSIVE INTRAVENOUS INFUSIONS

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The relation of oxygen lack to capillary permeability has been of considerable interest for many years. Landis (1927) found that brief periods of total ischaemia led to an increased permeability to protein in the capillaries of the mesentery of rats and frogs. Other workers, McMichael and Morris (1936), Stead and Warren (1944), were unable to demonstrate an increase in the capillary permeability to protein in the forearm and leg during more physiological degrees of anoxia. However, Henry, Goodman and Meehan (1947) concluded that with venous oxygen tensions below 15-20 mm. Hg., a significant increase in capillary permeability occurred in the capillaries of the forearm.

That the pulmonary capillaries were particularly vulnerable to oxygen lack has been more or less assumed since the work of Drinker and his colleagues (Warren, Peterson and Drinker, 1942; Drinker and Warren, 1943; Warren and Drinker, 1942; Drinker, 1945). These authors found that under conditions of controlled respiration there was an increase in the flow of lymph from the right lymph duct when the animals were exposed to gas mixtures containing 10 p.c. and 13·5 p.c. oxygen in nitrogen. On the basis of these findings the authors postulated that anoxia of this severity led to a selective increase in the permeability of the lung capillaries and was thus an important factor in the genesis of pulmonary oedema. In the above experiments, no post-mortem findings were given, so it was decided to re-investigate the part played by anoxia to ascertain whether pulmonary oedema actually occurred.

In the present experiments it was felt desirable to use a standard method for producing graded amounts of pulmonary oedema and to compare the ease of inducing the lung oedema when the animals were breathing air and low oxygen mixtures. As our standard method we adopted the use of massive intravenous infusions of Ringer-Locke solution, and whilst this method of producing lung oedema in normal animals is rather artificial, it was found useful in bringing out the differences referred to above. A series of experiments designed to investigate the effects of transfusion and anoxia on cardiac output, arterial and venous pressures and respiratory changes was also carried out.

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METHODS.

Rabbits varying in weight from 1·2–3·0 kg., mainly 1·5–2·0 kg., were used in all experiments. Most experiments were performed on unanaesthetized animals.

Administration of gas mixtures. When the rabbits were exposed to the low oxygen mixtures (11 p.c. and 13·5 p.c.), an airtight mask was applied to the rabbit's head, and it breathed the gas mixture from an anaesthetic bag kept half filled with the required mixture which was supplied from a cylinder. An open-circuit system of gas exchange was used and the rabbit breathed through small rubber valves of low resistance. The total dead-space of the valve arrangement was about 3–4 ml.

Transfusion. Unanaesthetized rabbits were transfused through the ear vein for periods of 5 hours with sterile Ringer-Locke solution, warmed so that the effluent temperature was about 32–34° C. A 23-gauge needle attached to a conventional blood transfusion apparatus was used to deliver the fluid at rates kept constant for each particular experiment. In earlier experiments the animals were kept in a rabbit box. It was later found convenient, however, to keep the rabbit in a bag or sling which was suspended from a metal frame. This bag was so constructed of soft leather that the chest movements were unimpeded and most of the weight of the animal was supported by the crutches of its limbs which emerged from holes in the side of the bag. The limbs were usually further supported by means of tapes attached to the metal frame. The animal would remain quiet and show no signs of distress in this arrangement for the period of the experiment, 5 hours.

Cardiac output. The direct Fick method was used to determine cardiac output. Rabbits were lightly anaesthetized with 20 mg. nembutal/kg. which produced mild narcosis, but no analgesia. The skin in the neck was infiltrated with 5–8 ml. of 1 p.c. procaine hydrochloride so that the operative work was done under light narcosis and mainly under local anaesthesia. The right external jugular vein was defined and a polyethylene catheter introduced into the right auricle or in some cases into the right ventricle. The catheter was filled with heparinized Ringer-Locke solution. A second catheter was introduced into the right carotid artery. Finally, an L-shaped tracheotomy tube was introduced into the trachea and the skin was closed by suturing. The tracheotomy tube was used here in preference to a mask to ensure that there was no leak when determining the oxygen consumption. Local anaesthetic (about 3–4 ml.) was injected around the skin sinus through which the tracheotomy tube passed. About 1–1½ hours was then allowed to elapse before the experiment proper commenced. At the end of that time the rabbit had emerged from the anaesthetic. It was then transferred to a rabbit bag and kept warm by means of a radiator.

Air analyses. Expired air was collected in a rubber bag, filled previously overnight with expired air to saturate the rubber with CO_2, through the valve arrangement described above. The volume of air was measured with a gas meter, both expired and inspired air samples were analysed with the Haldane gas analysis apparatus, and the oxygen consumption calculated. Duplicate collections of expired air were frequently made.

Blood analyses. Blood was collected through the two catheters into heavily paraffined syringes and was stored on ice under paraffin. The oxygen saturation of the blood was determined by the modified Haldane blood gas analysis apparatus (Courtice and Douglas, 1947). From the oxygen consumption and the A-V oxygen difference the cardiac output was calculated.

Venous pressure. This was determined through the ear vein, and in those experiments in which transfusions were given it was determined through the transfusion needle. In a few experiments this was compared to venous pressure in the external jugular vein and it was found that changes in the latter were reflected in the ear venous pressure. The method of recording was a slight modification of the method described by Burch and Winsor (1943).

Arterial pressures. These were recorded through a polyethylene catheter in the carotid artery by means of a Hürthle type of manometer.

Change in plasma volume following transfusions of Ringer-Locke. This was determined by noting the change in haemoglobin concentration during the experiment. In a few instances haematocrits were done in parallel with the haemoglobin estimations, and the change in blood volume calculated by the two methods agreed. The haemoglobin concentration was determined with an M.R.C. Grey-Wedge photometer.

The protein concentration in the plasma and in the effusions was determined by micro-kjeldahl digestion, nesslerization and estimation with a Hilger "Spekker" absorptiometer.

Method of killing animals and post-mortem examination. The rabbits were killed by the rapid intravenous injection of 80 mg. nembutal/kg. and the post-mortem was carried out immediately after death (Durlacher, Banfield and Bergner, 1950). Fluid present in the peritoneal, pleural and pericardial cavities was sucked off by means of Pasteur pipettes and measured. A ligature was tied around the trachea and the lungs were removed, weighed and examined for gross evidence of pulmonary oedema. In numerous instances histological examination of the lungs was carried out and it was found that the macroscopic and microscopic evidence of pulmonary oedema correlated satisfactorily.

RESULTS.

The lung-body weight ratio as a measure of pulmonary oedema.

It was felt desirable to have some quantitative measure of pulmonary oedema and in the course of our experiments it was found that the lung/body weight ratio (i.e. weight of the lung in gm. divided by body weight in kg.) correlated well with the gross post-mortem appearance of the lungs examined immediately after death. Four gradings of lung appearance were used: 0—when there was no pulmonary oedema; 1—when only congestive changes were present; 2—when there was moderate macroscopic uniform oedema; and 3—when there was marked macroscopic uniform oedema with tracheal frothing. The results are shown in Fig. 1. It is evident that the lung/body weight ratio rose quite strikingly as oedema accumulated in the lung and that the mean lung/body weight of each of the above categories differed significantly from that of the next highest grade.

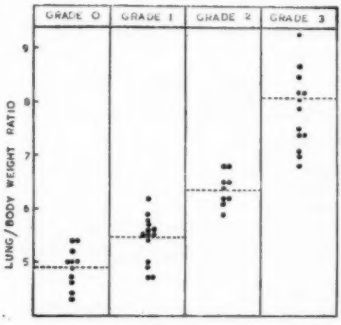

Fig. 1. Relation between the lung/body weight ratio (gm./kg.) and the grade of pulmonary oedema assessed from the macroscopic appearance of the lungs.

The effect of 5 hours' anoxia without transfusion on the production of pulmonary oedema.

Seven unanaesthetized rabbits were subjected to 11 p.c. oxygen in nitrogen for 5 hours. At the end of that time they were killed and the lungs were examined for evidence of pulmonary oedema. No pulmonary oedema was

found to be present in any case, the mean lung/body weight ratio being 5·0± 0·12. It appeared, therefore, that even this fairly prolonged exposure to anoxia did not by itself lead to pulmonary oedema in unanaesthetized rabbits.

Production of pulmonary oedema by means of intravenous Ringer-Locke solution when breathing air, 11 p.c. oxygen or 13·5 p.c. oxygen.

Rabbits were given a continuous drip transfusion of Ringer-Locke solution for 5 hours. The rate of transfusion was varied in different experiments, and the animal breathed throughout, air, 11 p.c. oxygen in nitrogen or 13·5 p.c. oxygen in nitrogen. Most of the animals were unanaesthetized, but a few were lightly narcotized with nembutal, the amount given in these cases being 40-60 mg./kg. over the 5 hour period. At the end of the 5 hour period, the amount of fluid transfused, the volume of urine passed, the amount of fluid retained in the tissues, the increase in plasma volume, the effusions in peritoneal, pleural and pericardial cavities and the lung/body weight ratio were measured or calculated. The mean results of all these experiments are given in Table 1.

TABLE 1.

Effect of transfusing rabbits with Ringer-Locke solution when breathing air, 11 p.c. or 13·5 p.c. oxygen in nitrogen. Mean figures in all cases.

	No. of rabbits	Weight kg.	Amount of fluid given ml./kg.	Amount of urine passed ml./kg.	Amount of fluid retained ml/kg.	Rise in plasma volume ml/kg.	Effusions ml./kg.	Lung/body wt. ratio gm./kg.	Total oedema ml./kg.
Air									
Unanaesthetized 5-hr. expt.	11	1·8	331±19	156±29	175	10±2	4·4±0·9	6·3±1·1	164±16
Anaesthetized 5-hr. expt.	5	2·1	417±32	236±41	181	8±2	2·8±0·7	5·9±0·45	163±40
Total 5-hr. expt.	16	1·9	358±38	181±38	177	9±2	3·9±1·1	6·1±1·3	164±22
11 p.c. oxygen									
Unanaesthetized 5-hr. expt.	8	1·6	170±18	64±12	106	11±1·5	1·7±0·4	6·5±0·3	95±7
Anaesthetized 5-hr. expt.	3	1·5	152±25	65±30	87	13±2·2	2·2±0·8	6·2±0·7	74±8
Total 5-hr. expt.	11	1·6	165±31	65±32	100	11±2·6	1·8±0·7	6·4±0·8	89±7
Unanaesthetized 2-hr. expt.	4	1·5	155±24	45±15	110	10±1·5	2·4±0·6	7·1±0·2	100±10
13·5 p.c. oxygen									
Unanaesthetized 5-hr. expt.	8	1·7	225±22	118±33	107	8±1·4	1·4±0·4	6·6±0·9	99±15

The figures given next to the means are the standard errors of the means. The increase in plasma volume has been calculated from the change in haemoglobin levels, and has been converted into absolute figures on the assumption that the normal rabbit plasma volume is 40 ml./kg. The "total oedema" refers to the excess fluid present in the body tissues and is the total amount of fluid given less the amount of urine passed and the absolute increase in plasma volume.

Since there was little difference in the behaviour of the unanaesthetized and lightly narcotized animals, results of these two groups have been pooled. The Table shows that in the anoxic animal considerably less fluid has to be infused to produce a similar degree of pulmonary oedema to that obtained in the normal animal. Thus with air, in order to produce a moderate degree of pulmonary oedema, lung/body weight ratio of 6·1, an average of 358 ml./kg. of Ringer-Locke solution was given. Of this amount 177 ml./kg. was retained in the body and was distributed as follows: About 9 ml./kg. of fluid remained in the plasma; about 4 ml./kg. went to the serous cavities (peritoneum, pleura and pericardium); about 1·2 ml./kg. accumulated in the lungs, and the remainder was distributed in the rest of the body tissues. With the animals breathing 11 p.c. oxygen the same degree of pulmonary oedema was produced, lung/body weight ratio 6·4, when only 165 ml./kg. was given. Of this 100 ml./kg. was retained in the body; 11 ml./kg. in the plasma; 1·8 ml./kg. in the serous cavities, 1·5 ml./kg. in the lungs, and the rest in the systemic tissues. The last column of the 11 p.c. oxygen section of the Table shows the results of experiments where rabbits were transfused at a faster rate. These rabbits all died after 2 hours, probably of cardiac failure. The total amount of fluid given to these animals was slightly less than that given to the 11 p.c. oxygen 5 hr. rabbits, and the degree of pulmonary oedema slightly greater than that found in the latter group. The experiments showed that when rabbits were breathing 11 p.c. oxygen the amount of pulmonary oedema produced was roughly the same for a given transfusion volume, whether this be given in 2 or in 5 hours, and that this volume was in all cases about half that required with air. In the 13·5 p.c. oxygen experiments the total fluid transfused to produce roughly the same degree of pulmonary oedema as occurred in the air experiments (lung/body weight ratio 6·6) was again significantly less (about 2/3) than with the air experiments.

In Fig. 2 the lung/body weight ratio has been plotted against the total excess oedema fluid which accumulated in the tissues during the 5 hr. period of intravenous infusion in individual experiments at various transfusion rates. The graph thus represents the proportion of fluid which accumulated in the lungs as compared to the rest of the body. When rabbits were breathing air very little fluid accumulated in the lungs as compared to the rest of the body until about 140 ml./kg. had been distributed in the tissues, i.e. a volume approximately equivalent to the normal volume of extra-cellular fluid. From that point onwards fluid steadily accumulated in the lungs and when the total excess tissue fluid was 200 ml./kg. a considerable degree of pulmonary oedema resulted. Of the final 60 ml./kg. about 2·3 ml./kg. appeared in the lungs.

With 11 p.c. oxygen, fluid began to accumulate in the lungs at a much lower infusion rate. Pulmonary oedema began to form when the total excess tissue fluid was only 50 ml./kg. and when this reached 120 ml./kg. there was a

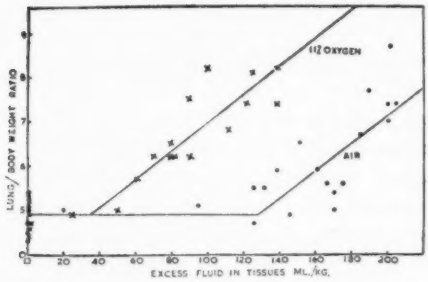

Fig. 2. Relation between lung/body weight ratio and total excess fluid in body tissues (''total oedema'').

● Rabbits breathing air.
× Rabbits breathing 11 p.c. oxygen in nitrogen.

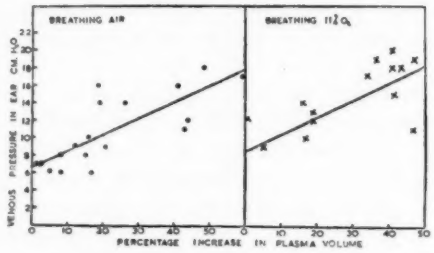

Fig. 3. Relation between venous pressure in ear vein and the percentage increase in plasma volume.

● Rabbits breathing air.
× Rabbits breathing 11 p.c. oxygen in nitrogen.

considerable degree of pulmonary oedema present. A similar state of affairs held for animals exposed to the 13·5 p.c. oxygen mixture, but the points have not been drawn in Fig. 2.

In Fig. 2 the linear regression lines expressing the relation between the lung/body weight ratio and the excess oedema fluid have been drawn. In view of the number of experiments these represent a reasonable approximation, although the true relationship between the variates is probably a curve. It is evident that the regression lines are parallel for experiments in which animals were exposed to air and 11 p.c. oxygen. The actual figures are summarized in Table 2.

TABLE 2.

The relation of the lung/body weight ratio to the total body oedema accumulating in the course of transfusions.

	Regression equation	Significance of regression coefficient	Mean lung/body weight ratio	Mean oedema ml./kg./5 hr.	Amount of oedema with lung/body weight ratio of 6·8 ml./kg./5 hr.
Air	L.B. = 6·08 + 0·032 (E.—164)	P = 0·005	6·08	164	186
11 p.c. oxygen	L.B. = 6·8 + 0·028 (E.—95)	P = 0·001	6·8	95	95
13·5 p.c. oxygen	L.B. = 6·6 + 0·031 (E.—99)	P = 0·015	6·6	99	105

L.B. = Lung/body weight ratio; E. = Oedema in ml./kg./5 hr.

From Table 2 it is clear that the regression coefficients are practically identical in experiments in which the rabbits were exposed to all three gas mixtures. This means that under all three conditions no greater proportion of fluid (about 3 p.c. in all cases) accumulated in the lungs once pulmonary oedema commenced as compared to the rest of the body. It was only with the

low oxygen mixtures that fluid accumulated at lower transfusion rates as was shown in Table 1. The last column of Table 2 shows that the same degree of pulmonary oedema resulted when nearly twice as much fluid had accumulated in the tissues with air as with the low oxygen mixtures.

The fact that with increasing infusions the same proportion of fluid accumulated in the lung compared to the rest of the body with all three gas mixtures suggests that even under the stress of overloading the circulation anoxia did not selectively influence the permeability of the lung capillaries to fluid.

The rise in plasma volume in relation to the venous pressure.

In Fig. 3 the percentage increase in plasma volume has been plotted against the venous pressure measured in the ear vein. Both with air and with 11 p.c. oxygen the venous pressure rose as the plasma volume increased. The data have been summarized in Table 3. The slopes of the regression lines are nearly parallel. This means that for a given increment of plasma volume, there was a similar increment of venous pressure when the animals were breathing air and 11 p.c. oxygen. For a given absolute plasma volume increase, however, the average level of venous pressure was higher when the animals were breathing the low oxygen mixture compared to air. Thus for a mean increase in plasma volume of 25 p.c. the mean venous pressure was 11·4 cm. H_2O with air and 14·0 cm. H_2O with 11 p.c. oxygen. This difference is statistically significant. As regards the actual level of the increase in the plasma volume in the two groups, the mean increase with air was 23 p.c. and with the low oxygen mixture 29 p.c. The Standard Error of the difference is 4 p.c. and the difference is not statistically significant.

TABLE 3.

Relation between percentage increase in plasma volume and venous pressure in ear vein.

	Regression equations	Significance of regression coefficient from zero	Significance of regression coefficients from each other	Venous pressures with increase in plasma volume of 25 p.c.	Significance of difference of venous pressures
Air	V.P. = 11 + 0·178 (P.V.—23)	P = 0·001		11·4 cm. ± 0·70	
			P = 0·55		P = 0·01
11 p.c. oxygen	V.P. = 14·7 + 0·165 (P.V.—29)	P = 0·005		14·0 cm. ± 0·71	

V.P. is Venous Pressure in ear vein. P.V. is percentage increase in plasma volume.

In the two groups of experiments then, very different transfusion rates produced roughly the same increase in plasma volume during the 5 hours' transfusion period, but the venous pressure was higher for a given absolute increase in plasma volume when rabbits were breathing the low oxygen mixtures.

The drop in colloid osmotic pressure with transfusion.

When the rabbits were breathing air, the mean plasma protein concentration before transfusion was 6·2±0·3 gm. p.c. At the end of transfusion it was 4·3±0·3 gm. p.c. There was thus a 30 p.c. reduction in the colloid osmotic pressure. With 11 p.c. oxygen, the mean plasma protein concentration before transfusion was 6·3±0·4 gm. p.c. and after transfusion 4·2±0·5 gm. p.c. There was again a reduction of about 30 p.c. in the colloid osmotic pressure. The drop in colloid osmotic pressure in the course of transfusion was thus the same when the rabbits breathed air or 11 p.c. oxygen.

Cardio-respiratory changes during anoxia and transfusion.

In order to ascertain the changes which occurred in the circulation during transfusion and anoxia, the cardiac output and the arterial and venous blood pressures were determined. The cardiac output measurements were obtained from a group of 12 rabbits—6 of these rabbits were breathing air and 6 animals were breathing 11 p.c. oxygen. Two animals in each group had no transfusion, two had a transfusion sufficient to give them moderate lung oedema and in two the transfusion was large enough to give them fairly severe pulmonary oedema. The amount of fluid required to produce a given degree of pulmonary oedema was roughly the same in these experiments with the cardiac catheter, as in the ordinary transfusion experiments. The results were analysed statistically in all instances by the analysis of variance technique (**Fisher, 1946**).

TABLE 4.

Cardiorespiratory changes during anoxia and transfusion in individual experiments.

	Cardiac output ml./kg.		Stroke volume ml./kg.		Heart rate/min.		Oxygen consumption ml./kg./min.	
	0 hr.	5 hr.	0 hr.	5 hr.	0 hr.	5 hr.	0 hr.	5 hr.
Air								
No transfusion	386	373	1·9	1·9	200	196	19·0	13·1
	233	281	1·3	1·4	180	200	15·5	19·4
Transfusion giving moderate lung oedema	230	550	1·2	2·2	190	244	11·4	23·2
	300	173	1·5	0·72	200	240	12·0	11·8
Transfusion giving severe lung oedema	237	278	1·2	1·1	190	250	14·7	12·3
	263	208	1·9	1·6	140	180	14·6	13·7
11 p.c. oxygen								
No transfusion	171	78	0·77	0·55	220	140	8·1	5·3
	166	71	0·81	0·39	204	180	8·1	3·1
Transfusion giving moderate lung oedema	207	111	0·95	0·65	216	170	7·1	10·4
	96	42	0·60	0·34	160	150	6·8	2·4
Transfusion giving severe lung oedema	280	83	1·1	0·43	250	190	10·7	4·2
	134	81	0·83	0·73	160	110	4·8	3·3

The individual determinations are given in Table 4. All variables were measured on two occasions. The zero time measurement in the case of air was taken after the rabbits had been in the rabbit bag for twenty minutes and before the transfusions were started. With the rabbits breathing 11 p.c. oxygen the zero time measurement was taken after the rabbits had been in the bag for 20-25 minutes and had been breathing 11 p.c. oxygen for 15-20 minutes. The second measurement was taken 5 hours later at the end of each respective treatment. The animals were sacrificed immediately following the second measurement and a post-mortem examination was immediately carried out in which the position of the catheters was checked and the lungs were examined.

Cardiac minute volume was significantly reduced following 15-20 minutes' exposure to 11 p.c. oxygen. Thus the mean cardiac output was 275 ml./kg. with air and 175 ml./kg. with 11 p.c. oxygen. There was some overlap in the two groups but the standard error of the difference between the means is 23·6, and the difference is statistically highly significant ($P = 0{\cdot}005$). At the end of 5 hours there was no significant change in cardiac output in the group of rabbits breathing air, but the cardiac minute volume was greatly reduced in the group breathing 11 p.c. oxygen in nitrogen. All these changes occurred whether the rabbits were transfused or not. We may thus conclude that the cardiac minute volume was significantly reduced in the rabbit following 15 minutes' anoxia, and this difference was further increased after 5 hours' anoxia. Transfusion did not appear to affect the cardiac minute volume to a significant degree.

The initial decreased cardiac output in anoxia was mainly attributable to a fall in stroke volume. The mean stroke volume at the beginning of the experiment was 1·5 ml./kg. for the six animals breathing air and 0·83 ml./kg. for the six breathing 11 p.c. oxygen. This is a significant reduction. After 5 hours' transfusion in the four animals breathing air and in the four breathing 11 p.c. oxygen there was in general a further fall in stroke volume.

The heart rate showed little change over the 5 hour period when the animals were breathing air without a transfusion. With air and transfusion there was a significant cardio-acceleration of an average of 50 beats/minute. With 11 p.c. oxygen there was at first a suggestion of cardio-acceleration when compared to air while at the end of 5 hours there was a significant reduction of an average of 40 beats/minute heart rate in all rabbits whether transfused or not.

It was not possible to determine blood pressures and cardiac output simultaneously. For this reason venous pressures were determined at various times throughout the initial series of experiments, while the mean arterial pressures were measured in four groups of animals, two in each group, breathing air and

11 p.c. oxygen without transfusion for 5 hours and breathing air and 11 p.c. oxygen with a transfusion leading to a severe degree of pulmonary oedema in 5 hours. The composite results are shown in Fig. 4.

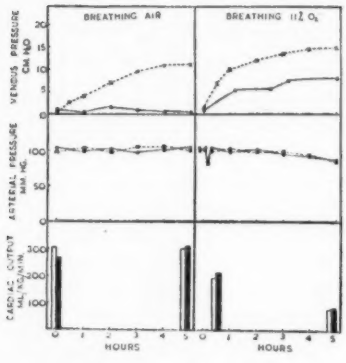

Fig. 4. The venous and arterial pressures and cardiac output in rabbits over a period of 5 hours breathing either air or 11 p.c. oxygen, with and without transfusion.

●——● No transfusion.
×-----× Transfusion.
□ Cardiac output, no transfusion.
■ Cardiac output, transfusion.

The mean arterial pressure showed little change except for minor fluctuations when the animals were breathing air and did not change with transfusions. When rabbits were placed on the 11 p.c. oxygen mixture, there was first of all a drop of about 20 mm. Hg. lasting about 30-40 seconds and the blood pressure then returned to its normal value of about 100 mm. Hg. Subsequently it declined very gradually during the remainder of the five hour period, and at the end of that period reached a level of about 85 mm. Hg. Since the cardiac output was lower with anoxia compared to air and the mean arterial pressure was at first unaltered, there must have been a rise in the peripheral resistance in the systemic circulation. The blood pressure was thus probably maintained at its control level by means of systemic vasoconstriction. At the end of the 5 hour period there was a fall in cardiac output of over 50 p.c. with a fall in arterial pressure of only about 15 p.c., so that presumably the vasoconstriction became even more intense so as to maintain the blood pressure at a reasonable level.

The venous pressure in animals breathing air without transfusion showed no change during the 5 hour period of observation but there was a definite rise in venous pressure in the transfused animals. With anoxia the venous pressure rose even when no transfusion was given, and rose further during transfusion. The rise in venous pressure with anoxia under all conditions was always significantly higher than with air.

The mean resting oxygen consumption of the animals breathing air was 14·8 ml./kg./minute, whilst that of the rabbits exposed to 11 p.c. oxygen for 15-20 minutes was 7·6 ml./kg./minute, the difference being highly significant. Transfusion did not appear to affect the oxygen consumption. Thus at the end of 5 hours the mean oxygen consumption of the animals breathing air was 15·6 ml./kg./minute, whilst that of the animals exposed to the 11 p.c. oxygen mixture had dropped further in five out of six cases from the initial low level

and now averaged 4·8 ml./kg./minute. The drop in oxygen consumption occurred gradually through the period of exposure to the low oxygen mixture, though it was somewhat more rapid during the last hour's exposure. It occurred in spite of an adequate ventilation and so probably represents a true reduction in the metabolism.

TABLE 5.

Arterial and venous oxygen saturations in individual experiments with transfusion and oxygen lack.

	p.c. saturation of haemoglobin with oxygen			
	Arterial blood		Mixed venous blood	
	0 hr.	5 hr.	0 hr.	5 hr.
Air.				
No transfusion	94	96	62	61
	96	98	66	74
Transfusion giving moderate lung oedema	99	96	69	43
	99	99	70	66
Transfusion giving severe lung oedema	98	89	61	35
	98	91	58	50
11 p.c. oxygen				
No transfusion	58	62	26	23
	67	60	40	11
Transfusion giving moderate lung oedema	77	77	60	24
	62	64	20	13
Transfusion giving severe lung oedema	66	54	45	16
	52	37	36	13

The arterial and mixed venous oxygen saturation are summarized in Table 5. Only transfusions which led to the severer grades of pulmonary oedema caused a significant decrease in the arterial oxygen saturation over the 5 hour experimental period. The decrease in the saturation of the mixed venous blood probably depended in part on the rise of venous pressure, which accompanied anoxia and transfusion (including transfusions with air) and partly on the diminished oxygen capacity of the blood caused by the transfusion.

DISCUSSION.

Anoxia and capillary permeability.

The experiments showed that exposure to 11 p.c. oxygen for 5 hours did not by itself lead to pulmonary oedema in rabbits. Since extravascular protein is only slowly removed from the lungs (Drinker and Hardenbergh, 1947; Courtice and Simmonds, 1949), it is probable that anoxia of this degree in rabbits did not lead to any gross alteration of capillary permeability.

The only difference in the development of pulmonary oedema in the transfused animals breathing 11 p.c. oxygen and in those breathing air was that pulmonary oedema began to appear at a lower transfusion rate in the anoxic animals. Once oedema appeared, however, the proportion of fluid escaping in the lungs compared to that accumulating in the systemic tissues was the same whether air or 11 p.c. oxygen was breathed. Since the osmotic pressure of the plasma proteins is normally about 25 mm. Hg. and the pulmonary capillary pressure only 5-10 mm. Hg. (Doyle, Wilson, Estes and Warren, 1951; Dexter, Dow, Haynes, Whittenberger, Ferris, Goodale and Hellems, 1950), there is in the lesser circulation a considerable reserve of pressure in favour of reabsorption of fluid. The experimental results could be explained, therefore, on the basis that in both the normal and the anoxic animal pulmonary oedema was produced when the capillary pressure rose to overcome the osmotic pressure reserve, and that in overloading the circulation with fluid this point was reached sooner in the anoxic animals. Once this point was reached, however, a given amount of infused fluid would increase the pulmonary vascular volume and pulmonary capillary pressure to approximately the same extent whether air or 11 p.c. oxygen was breathed, so giving rise to the same increment of pulmonary oedema in each case. This interpretation suggests that anoxia does not cause a selective increase in permeability of the lung capillaries compared with that of the capillaries of the rest of the body. Since the balance of experimental evidence indicates that this degree of anoxia does not affect the permeability of the systemic capillaries, this means that the pulmonary capillary permeability is not increased.

In the light of these findings the results of Drinker and his associates are of interest. These workers found that when anaesthetized dogs were subjected to 10 p.c. oxygen in nitrogen and 13·5 p.c. oxygen in nitrogen, there was an immediate increase in the lymph flow from the right lymph duct, draining mostly the lungs. On the basis of these findings the above workers concluded: "If anoxia is accompanied by a greater flow in lung lymph and the cardiac output remains the same as in the control period or falls, then, given absolutely constant lung movement during the entire experiment it is fair to attribute increased lymph flow to increased permeability of the lung capillaries arising from anoxia." These experiments could, however, also be explained by an increase in capillary pressure leading to an increasd capillary filtrate with a resultant fall in lymph protein concentration, which actually occurred. A similar picture as regards lymph flow was obtained by Warren and Drinker (1942) when they partially clamped the pulmonary veins of the dog, and in these experiments increased filtration most probably played a major rôle. It would appear, then, that these experiments do not necessarily prove that anoxia leads to an increase in pulmonary capillary permeability.

Anoxia and cardiac output.

If these effects of anoxia are to be explained by an increase in pulmonary capillary pressure, the cause of the increased pressure should be determined. The circulatory findings in the present series indicated that anoxia gave rise to peripheral vasoconstriction in the systemic circulation to maintain the blood pressure with a falling cardiac output. This redistribution of blood would increase the volume of blood both in the venous side of the systemic circulation causing a rise in venous pressure, and in the expansile pulmonary vascular bed where there may or may not be a significant increase in capillary pressure (Cournand, 1950; Courtice, 1953). Regarding the effect on pulmonary pressures, many workers have found that short periods of anoxia cause a rise in the arterial pressure (v. Euler and Liljestrand, 1947; Lewis and Gorlin, 1952; Doyle *et al.*, 1952; Motley, Cournand, Werko, Himmelstein and Dresdale, 1947). The pulmonary venous pressure or left atrial pressure measured in anaesthetized animals has been shown to change but little. There is no definite evidence concerning the behaviour of the pulmonary venous pressures following prolonged anoxia.

There is, however, quite a variety of opinion in the literature on the effect of acute anoxia on the cardiac output using the direct Fick method. Thus in man Motley *et al.* found a slight reduction in cardiac output following ten minutes of breathing 10 p.c. oxygen, but later Cournand (1950) found that about 20-30 minutes breathing 10 p.c. oxygen led to an increased cardiac output if the arterial oxygen saturation fell below 80 p.c. In animals the results are somewhat variable. Drinker *et al.* (1942) found that breathing 10 p.c. oxygen in anaesthetized dogs for 1-3 hours led to a 50 p.c. reduction in cardiac output, but Doi (1921) working with anaesthetized cats found that breathing 9-11 p.c. oxygen mixtures for 2-3 hours led either to no change in cardiac output or a slight decrease. Harrison and Blalock (1927), using a rebreathing method in unanaesthetized dogs, in which a steady state was never reached as the oxygen concentration in the spirometer was constantly declining and which thus rather invalidates the Fick principle (Cournand, 1950), found that short periods (up to 20 minutes) exposure to milder anoxia led to no change in the cardiac output, and very severe anoxaemia with arterial oxygen saturations below 40 p.c. led to an increase in cardiac output. Maybe some of the variations in these results are due to anaesthesia, the species of animal used and the degree and duration of anoxia. In the rabbit deep nembutal anaesthesia greatly decreased the percentage saturation of the arterial blood and the cardiac output. For this reason all the experiments in the present series were performed in the unanaesthetized animal in which the mean resting cardiac output while breathing air was 275 ml./kg. Leegaard (1927), however, found that the mean cardiac output was 110 ml./kg. in albino and 150 ml./kg. in brown rabbits anaesthetized with urethane. It is also probable that in anoxia the redis-

tribution of blood by the action of the vasomotor centre will differ in degree in the unanaesthetized and anaesthetized rabbit and also in different species of animals.

Rgarding the change in oxygen consumption following acute anoxia Motley *et al.* (1947) found a slight decrease in the oxygen consumption of man following 10 minutes' exposure to 10 p.c. oxygen. Lintzel (1931) and Blood, Elliott and D'Amour (1946) found that the metabolism and body temperature of rats declined at simulated altitudes exceeding 20,000–25,000 feet. Kottke, Phalen, Taylor, Visscher and Evans (1948) found a reduction in body temperature in mice and dogs exposed to anoxia for several hours and noted that anoxia prevented an increase in the oxygen consumption when these animals were exposed to cold; they found the same effect to a lesser degree in man. Finally, Walters (1927) found that prolonged exposure of rats to 0·02–0·05 p.c. carbon monoxide for 5-6 hours depressed their oxygen consumption. It appears, then, that acute anoxia can depress the oxygen consumption in several species and this is usually accompanied by a fall in body temperature.

Anoxia and transfusions.

If it is true, then, as the present experiments suggest, that in the unanaesthetized rabbit anoxia leads to cardiac failure with redistribution of blood, so increasing the pulmonary blood volume, the experiments with infusions can be more easily interpreted.

In men and in animals it has been shown that rapid intravenous transfusions increase the pulmonary capillary pressure (Doyle, Wilson, Estes and Warren, 1951; Gibbon, Gibbon and Kraul, 1943; Haddy, Campbell and Visscher, 1950). The resultant increase in blood volume causes an increase in the volume of the pulmonary vascular bed, but because of the expansibility of these vessels and of the reserve of osmotic pressure, only very considerable and prolonged transfusions of non-protein electrolyte solutions will raise the pulmonary capillary pressure sufficiently to cause gross oedema.

If in anoxia the pulmonary blood volume is already increased before transfusion, even though the capillary pressure is not significantly raised, it can readily be understood how a smaller transfusion than in the normal animal will suffice to distend the pulmonary vascular bed sufficiently to cause the capillary pressure to become greater than the osmotic pressure of the plasma proteins. The fall in colloid osmotic pressure which occurred during transfusion of the Ringer-Locke solution, though obviously a factor in allowing for the greater ease of filtration, was the same in both groups of experiments.

The effect of anoxia in precipitating pulmonary oedema in unanaesthetized rabbits receiving large and prolonged transfusions of Ringer-Locke solution can therefore be explained merely by altered haemodynamics without postulating an alteration in the permeability of the pulmonary capillary membrane.

SUMMARY.

The effect of anoxia on the production of pulmonary oedema following intravenous infusions of Ringer-Locke solution has been investigated in unanaesthetized rabbits. Breathing 11 p.c. oxygen in nitrogen for 5 hours did not produce pulmonary oedema.

With rabbits breathing gas mixtures containing 11 p.c. and 13·5 p.c. oxygen in nitrogen about half the amount of Ringer-Locke solution was required to produce a given degree of pulmonary oedema, compared to the amount required when the animals were breathing air.

The chief circulatory difference observed in rabbits breathing 11 p.c. oxygen compared with those breathing air was a reduction in cardiac output with a compensatory vasoconstriction to maintain the systemic blood pressure.

The resultant redistribution of blood in anoxia was thought to increase the amount of blood in the venous system, thus causing an increase in the venous pressure. The amount of blood in the pulmonary vascular bed would also be increased, so that smaller transfusions were required to raise the pulmonary capillary pressure sufficiently to overcome the osmotic reserve pressure. Once this point was reached, however, further infusions would cause the same degree of pulmonary oedema in the normal and anoxic animal.

The effect of anoxia in these experiments can, therefore, be explained by haemodynamic changes without postulating a selective increase on the pulmonary capillary permeability.

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THE ABSORPTION OF MONOSACCHARIDES FROM THE GASTROINTESTINAL TRACT OF THE GUINEA-PIG

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The digestive tracts of herbivora appear to be of two main types. Firstly that of the ruminant, in which fermentation with production of fatty acids takes place in the rumen prior to the foodstuffs entering the true stomach and small intestine so that by the time the food passes into the large intestine all significant absorption has been completed. The main function of the large intestine in this case appears to be abstraction of water. Secondly, those of the group represented by horse, guinea-pig, rabbit and others which, although living and thriving on the same diet as ruminants, have no rumen but possess a large caecum in which, it is presumed, fermentation proceeds in much the same manner as in the rumen. In this case it seems logical to expect provision for absorption from either the caecum or colon or both. For investigating the functions of the caecum and colon one of this group of animals, the guinea-pig, has been selected because it is easy to work with and readily procurable.

As it has been shown that monosaccharides are not absorbed from the large intestine of either the carnivorous dog (McNealy and Williams, 1929) or the omnivorous rat (Davidson and Garry, 1939), the first group of experiments in this investigation was devoted to studying the absorption of monosaccharides from the large intestine of the guinea-pig.

The investigation involved the introduction of glucose, fructose, galactose and xylose into isolated intestinal segments of the guinea-pig with their subsequent recovery and estimation; and the determination of blood glucose levels before and after introducing glucose into segments of the gastrointestinal tract.

METHOD.

Sodium pentobarbital anaesthesia was administered intraperitoneally.

The segment of intestine being studied was cut at either end, washed through with physiological saline until the washings were quite clear, then firmly ligated at both ends. The isotonic solution of monosaccharide was introduced from a burette directly through a fine hypodermic needle inserted through the wall of the gut until the segment was just filled without overdistention. After 2 to 2½ hours the intestinal segments were removed, opened and

[1] This work was done with the aid of a grant from the Commonwealth Research Fund for Universities.

washed thoroughly with distilled water, the washings were made up to volume and determinations of the respective sugars made by the Somogyi-Nelson method. The difference between amount of monosaccharide introduced and that recovered was taken as a measure of the quantity absorbed. Absorption of glucose from the caecum was also investigated on phlorrhizinized animals. On two occasions the caecum was only filled to about a sixth of its capacity, 3 ml. of isotonic glucose solution being introduced into a caecum of 20 to 30 ml. capacity. The absorption of hypertonic solutions of glucose from the colon was also investigated, using solutions of 10 p.c., 20 p.c. and 40 p.c. glucose.

In addition to the recovery experiments, blood sugar levels were determined ½ and 1 hour after introducing glucose into unwashed segments of the gastrointestinal tract. Five control animals received water into various sections of their intestine. The blood was obtained by cardiac puncture.

RESULTS.

Although the four monosaccharides were readily absorbed from isotonic solutions introduced into the small intestine, glucose alone was absorbed from the caecum and then only from the filled caecum.

TABLE 1.

The absorption in mg. of monosaccharides during 2–2½ hours following their introduction into gut of guinea-pigs.

	Small intestine		Colon		Caecum	
	Introduced	Difference*	Introduced	Difference*	Introduced	Difference*
Glucose 5·4 p.c.	285	—125	318	— 3	848	—273
			159	+ 3	1,512	—342
					162†	+ 3
					162†	+ 3
Glucose 2·5 p.c.					482	—107
Glucose 5·4 p.c. (after phlorrhizin)	238	—145			1,150	—150
	232	—128			1,150	—190
Glucose 10 p.c. ‡			105	+ 5		
			290	— 10		
Glucose 20 p.c.			400	— 25		
Glucose 40 p.c.§			1,600	—740		
			800	—330		
Xylose 4·5 p.c.	162	—111	126	— 6	945	+ 5
	320	— 60	130	— 2	840	— 10
Fructose 5·4 p.c.	270	—145	162	+ 7	540	+ 20
	265	—141	116	— 6	1,300	— 40
Galactose 5·4 p.c.	270	—110	228	— 8	830	0
	270	—116	275	+ 5	2,160	+ 20
					1,000	— 20

* Difference between amount introduced and that recovered.
† In these two cases the caecum was filled to only about one-sixth its capacity.
‡ 10 p.c. glucose left in colon for 5 hours before recovery.
§ 40 p.c. glucose left in colon for 4 hours before recovery.

The absorption of glucose from the caecum was rapid and comparable to its absorption from the small intestine. It was not inhibited by phlorrhizinization. None of the sugars was absorbed from isotonic solutions introduced into the colon, nor from 10 p.c. or 20 p.c. solutions of glucose, even after 5 hours; however, considerable absorption took place from a 40 p.c. glucose solution introduced into the colon.

As can be seen from Table 2, in all except one case, the blood glucose level, following the injection of glucose into small intestine caecum or colon, rose distinctly above that of control animals and even in this one case the reading half an hour previously was well above those of controls. However, in control animals, following the injection of water into various intestinal segments, the blood glucose rose considerably and on two occasions above that of an animal into whose intestines glucose had been injected.

TABLE 2.

Blood sugar levels following introduction of glucose into sections of the intestinal tract.

Site of injection of	amount in gm.	volume in ml.	Blood sugar in mg p.c. before injection	after ½ hour	after 1 hour
Water alone					
Caecum		7	116	122	148
Stomach		3	142	160	150
Duodenum		3	126	140	172
Caecum		2½	164	170	190
Glucose					
Caecum	3	7	100	186	250
Caecum	2	5	130	226	*156*
Colon	2	6	96	210	204
Small intestine	2	5	176	340	270
Small intestine	1	3	134	204	280

DISCUSSION.

The ready absorption of monosaccharides from the small intestine is what might be expected from previous work.

The absorption of glucose and failure of absorption of other monosaccharides from the caecum appears to be an interesting instance of a highly selective absorptive mechanism, in which also a hydrostatic factor appears to be involved as indicated by lack of glucose absorption from the partly filled caecum.

The failure of the colon to absorb glucose from isotonic solution was in conformity with findings in the rat and dog. Absorption of glucose from a 40 p.c. solution is comparable to the findings of Morrison, Shay, Rabdin and Cahoon (1939), that glucose absorption from the stomach of the dog was

possible from solutions of 40 p.c. of greater concentration. It may well be that in both cases the highly hypertonic solution so damages the mucous membrane that diffusion occurs directly into the tissues of stomach or colonic wall.

The rise of blood glucose in animals into segments of whose intestine water had been injected (Table 2) could probably be due to adrenalin secretion consequent on operative interference and handling of the intestines or even the previous cardiac puncture. The small rise in blood glucose levels reported in humans following rectal administration of glucose might well have some similar explanation.

SUMMARY.

Experiments involving the introduction of isotonic solutions of glucose, galactose, fructose and xylose into the isolated small intestine, caecum and colon of guinea-pigs and recovery of the sugar after 2 hours indicate that (a) all four monosaccharides are readily absorbed from the small intestine. (b) Of these monosaccharides glucose alone is absorbed and readily so from the caecum and this absorption is unaffected by phlorrhizin. (c) No absorption of monosaccharides occurs from isotonic solutions introduced into the colon.

Glucose is not absorbed from 10 p.c. or 20 p.c. solutions introduced into the colon but absorption occurs from a 40 p.c. solution.

The blood glucose level rises well above that of controls following the injection of strong glucose solutions into the small intestine, caecum or colon of the guinea-pig but the rise in blood glucose of control animals is quite marked.

Acknowledgment. I wish to record my appreciation of the continued interest and advice of Professor H. J. G. Hines throughout the performance of these experiments.

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THE INFLUENCE OF OXYGEN TENSION ON RESPIRATION AND CYTOCHROME a_2 FORMATION OF *ESCHERICHIA COLI*

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The experiments described in this paper arose as a result of information gained during attempts to prepare large quantities of *Escherichia coli* for the purpose of investigation of cytochrome a_2, and if possible to increase the cytochrome a_2 content of the bacteria. It is generally held that cytochrome a_2 has the function of a cytochrome oxidase. Carbon monoxide shifts the absorption band to 634 mμ, oxygen to 645 mμ, and aeration in the presence of cyanide causes the band to disappear.

Further evidence for the oxidase function of cytochrome a_2 of *Aerobacter aerogenes*, which has a similar cytochrome absorption spectrum to *E. coli*, was provided by Tissières (1951) who showed that on passing air into a cooled suspension of *A. aerogenes* cytochrome a_2 is oxidized before b_1, and on cessation of aeration cytochrome b_1 is reduced before a_2. However, Tissières has also shown that oxygen uptake is not directly related to the intensity of the a_2 absorption band in *A. aerogenes*, and suggested that cytochrome a_1 may also function as cytochrome oxidase. The same worker (1952) was able to prepare a cell-free suspension of particles, containing cytochrome a_1 and b_1 but no visible amount of cytochrome a_2. This suspension showed considerable respiratory activity.

The adaptive development of the cytochrome system of yeast has now been reported by a number of workers (Chin, 1950; Ephrussi, Slonimski and Perrodin, 1950; Ephrussi and Slonimski, 1950).

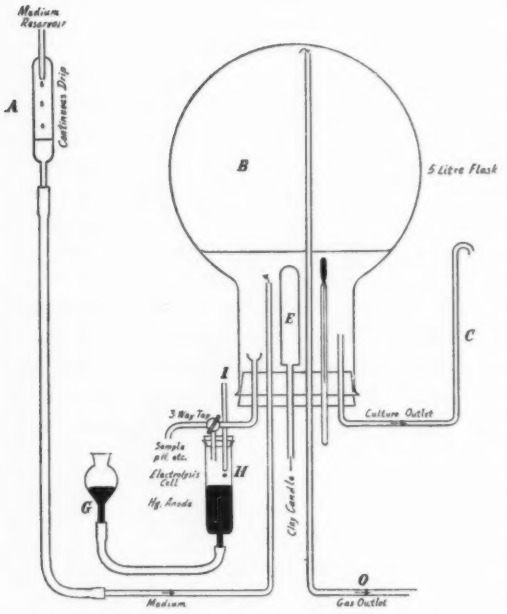
Schaeffer (1950) described an increase in the cytochromes of *Bacillus cereus* on aeration, and found that the medium from anaerobic culture contained much more free porphyrin than medium from aerobic culture. This suggested that porphyrins not used in the synthesis of cytochrome were excreted. In the case of *E. coli*, however, very little free porphyrin was found in the medium from anaerobic cultures, and none in aerobic culture.

Adaptive formation of the cytochromes of *E. coli*, and increase of respiration in the presence of oxygen was also found by the author, independently.

[1] This work has been carried out by the aid of a grant from the National Health and Medical Research Council of Australia.

Materials.

The organism. The strain of *E. coli* used in this experiment was obtained from the diagnostic laboratory of the hospital. The organism showed the following properties: indole was not formed; M.R. positive; V.P. negative; citrate was not used as a sole source of carbon; H_2/CO_2 ratio, 1:1; acid and gas from glucose, lactose, maltose, mannitol; sucrose, dulcitol, and salicin were not fermented; gelatin was not liquified; catalase was present. This strain developed a particularly strong cytochrome spectrum on aeration.



The medium consisted of: KH_2PO_4, 1·0 gm.; $(NH_4)_2HPO_4$, 4·0 gm.; sodium citrate, 2·0 g.m.; $MgSO_4.7H_2O$, 0·7 gm.; NaCl, 1·0 gm.; $FeSO_4.7H_2O$, 0·125 gm.; tap water to one litre. Sterile glucose to make 0·5 p.c. and veal broth 5 p.c. were added after autoclaving the salt solution.

Apparatus.

The continuous culture apparatus is shown in the Figure. Medium from a 10-litre reservoir was admitted to the 5-litre flask B through the drip A. Rate of influx of medium was measured by counting drops of known volume. C is an overflow which maintained a constant volume of 1 litre. Various mixtures of 5 p.c. CO_2 in O_2 and 5 p.c. CO_2 in N_2, were admitted by a fine grade Berkefeld candle E, which, under 30-lb. pressure, produced a vigorous turbulence by very small bubbles. Unabsorbed gas escaped by the vent O. Samples of culture were admitted to the electrolysis cell of a polarograph H by lowering the mercury bulb G. This mercury served also as the anode. The volume of the sample was 10 ml. This was admitted in 10 seconds. 250 ml. of inoculum grown under N_2 were admitted anaerobically from a flask (not shown in Figure) by a positive pressure of nitrogen. Temperature was maintained at 37° C. by keeping the temperature of the incubator at 40° C., and that of the reservoir (which was the only part of the apparatus not in the incubator) at 39° C. by a pilot flame. The polarograph was of the simple manual type. The dropping mercury electrode (I) was constructed from a clinical thermometer. Sensitivity of the galvanometer was 1,100 mm. per microamp., with the scale at 1 metre. The period was 2 seconds. The calomel reference cell and salt bridges contained KCl saturated at 32° C. An E.M.F. of 0·5 V. was applied cathodically; this was the midpoint of the first wave of oxygen in the medium. Galvanometer deflections were measured for oxygen-saturated, and oxygen-free, solutions before each experiment. The difference was taken as proportional to the concentration of oxygen in solution. This was found to be a reliable measure of oxygen tension by

the method of Lewis and McKenzie (1947): A saturated solution of oxygen in bacteria-free medium is mixed in various proportions with oxygen-free medium. The galvanometer deflection at 0·5 V. applied potential is found to be proportional to the amount of oxygen-saturated solution present.

EXPERIMENTAL PROCEDURES.

The problem of maintaining constant the rate of oxygen absorption during growth was overcome by a method of continuous dilution, while keeping the volume constant. The rate of dilution was such that bacterial density was kept constant.

This method of continuous culture was devised independently from and simultaneously to that by Monod (1950). Monod also described the mathematics and uses of the method.

Initially, oxygen tension and oxygen uptake rates were measured amperometrically as described by Skerman and Millis (1949) and Wise (1951).

However, it was found that Q_{O_2} measured by conventional manometry did not agree with that found amperometrically; consequently Q_{O_2} was measured by conventional manometry in this experiment (see polarographic measurement of Q_{O_2}).

5 p.c. CO_2 gas mixtures were used for aeration in all experiments. It was thought that the difference between amperometric and manometric results may be caused by the presence of CO_2 in the amperometric sample. Consequently manometry was also carried out in 5 p.c. CO_2 atmospheres by the use of CO_2 buffers. Oxygen tension during aeration was arrived at by a combination of manometric and amperometric methods.

The rate of solution of oxygen is equal to a (G—S), where G is the partial pressure of oxygen in the gas phase and S is the oxygen tension of oxygen in the solution. a is a constant dependent on the apparatus.

The rate of removal of oxygen by bacteria is qm, where m is the concentration of bacteria in mg. dry weight per ml. and q is the rate of oxygen uptake, here expressed in terms of decrease in oxygen tension per minute per mg. dry weight.

$$\frac{ds}{dt} = a(G—S)—qm \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad 1$$

The solution of this equation is:

$$S = (G—mq/a) \ (1—e^{-at}) \quad . \quad . \quad . \quad . \quad . \quad 2$$

The constant a was determined experimentally from an oxygen tension-time curve, obtained by measurement of oxygen tension amperometrically by passing oxygen into *sterile* oxygen-free medium. For the apparatus used here a had the value 1·75 min.$^{-1}$.

Oxygen tension during aeration of culture was then found as follows:

The expression $(1—e^{-at})$ was taken as 1, since equilibrium was established within a few minutes.

$$q = \frac{Q_{O_2}/60}{\text{solubility of } O_2 \text{ at } 37^\circ \text{ C.}}$$

The solubility of oxygen in the medium at 37° C. was determined by the Van Slyke apparatus, and was found to be 23 cmm. per ml. Q_{O_2} is given its conventional meaning.

From equation 2 it follows that

$$S = G - \frac{Q_{O_2} \times m}{60 \times 23 \times 1{\cdot}75}$$

$$= G - {\cdot}000414\, Q_{O_2}\, m$$

The rate of influx of medium which was required to maintain constant bacterial density was found in the following manner:

The required condition is that

$$\frac{dm}{dv} = \frac{m}{v} \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad 3$$

where m is the required mass of bacteria present in volume v in the aeration flask.

Now

$$\frac{dm}{dt} = km \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad 4$$

where k is the specific growth rate.

From equation 1:

$$\frac{dm}{dt} \cdot \frac{dt}{dv} = \frac{m}{v}$$

From this by substitution from equation 4:

$$\frac{dv}{dt} = kv \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad 5$$

The specific growth rate k was found from the formula

$$k = \frac{\ln\, m_2/m_1}{t_2 - t_1} \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad 6$$

which is derived by solution of equation 4 between time t_1 and t_2 when density is m_1 and m_2 respectively. This growth rate of anaerobic culture increased on aeration until a constant value was reached, in a period which was dependent on the oxygen tension. Thus it was necessary to adjust the rate of influx of medium at frequent intervals until a steady state had been reached.

The initial rate of influx was estimated from equations 5 and 6 by measurement of the growth rate of an anaerobic culture in the aeration flask prior to commencement of aeration.

Manometry.

This was carried out in conventional Warburg manometers containing air, and simultaneously in manometers fitted with Dixon-Simer cups with 5 p.c. CO_2 buffered atmospheres as described by Krebs (1951).

It was assumed that growth commenced immediately when bacteria were transferred from the aeration flask to the manometer, and that the specific growth rate remained constant during the forty-minute period of measurement. Q_{O_2} was calculated as follows:

By solution of equation 4: $m = m_O e^{kt}$. By suitable choice of units, the oxygen uptake rate of culture in the manometer is equal to $Q_{O_2}.m_O e^{kt}$.

From this

$$Q_{O_2} = \frac{\triangle O_2 k}{m_o} \quad \left(\frac{1}{e^{kt}{}_2 - e^{kt}{}_1}\right)$$

$\triangle O_2$ is the observed oxygen uptake, t_1 is the period from commencement of equilibration to closing the manometer taps. t_2 is the period from the commencement of equilibration to the end of measurement.

Oxygen tension in the culture contained in the manometers was of the same order as that in corresponding culture in the aeration vessel. This was shown by increasing the bacterial density of culture in the manometer flask until the oxygen uptake became independent of further increase of bacterial density at a certain shaking rate which was used throughout. These flasks contained air. From this the constant a of equation (1) applied to the manometer flask was found to be 2·2 min.$^{-1}$.

Polarographic measurement of Q_{O_2}.

In general Q_{O_2} measured amperometrically was lower than that measured by manometry. This was most noticeable when p_{O_2} during culture was high, in which case values as low as 40 p.c. of manometric values were obtained at the commencement of aeration. After 4½ hours, however, Q_{O_2} measured by both methods were usually similar.

Amperometric p_{O_2}–time curves for culture grown below 0·5 atm. were linear. However, when above 0·5 atm. the rate of oxygen uptake increased noticeably with falling p_{O_2}. Q_{O_2} in this case was calculated from the straight line of best fit.

It sometimes happened that points on the graph were so irregular that a smooth curve could not be constructed.

The difference between manometric and amperometric measurements may be due to the fact that invisible gas bubbles were withdrawn into the electrolysis cell, or that respiration was inhibited at high p_{O_2} from oxygen poisoning or from the formation of hydrogen peroxide. The first explanation is thought to be the more likely.

General procedure.

The amount of oxygen in the gas mixture necessary to give a certain oxygen tension was found amperometrically beforehand, by passing the gas mixture through *sterile* medium in the aeration flask and adjusting until the calculated galvanometer deflection was obtained. Gas was delivered from two cylinders containing 5 p.c. CO_2 in O_2 and 5 p.c. CO_2 in N_2. The main valves of the cylinders were opened fully and adjustments made by alteration of the fine adjustment valves. The main valve of the nitrogen cylinder was then shut off, and the galvanometer deflection for oxygen determined when the reading became steady again. The deflection corresponding to residual current was determined by shutting off the main valve of the oxygen cylinder and opening that of the nitrogen cylinder.

To 750 ml. of sterile oxygen-free medium contained in the aeration flask after adjusting the gas mixture, 250 ml. of anaerobic culture were added anaerobically. The added culture was prepared from a needle point inoculum grown at 37° C. for fourteen hours. This large inoculum ensured that sufficient essential metabolites were added, and that inhibitory substances were not present in amounts sufficient to limit growth.

Aeration and influx of medium were not commenced until the optical density of a sample indicated a bacterial content of 0·15 mg. per ml. Density was measured at 15 minute intervals during this period. The specific growth rate was calculated from these measurements by use of equation 6, when growth was established. The initial rate of influx was estimated from equation 5. This was usually about 6 ml. per minute, whereas 8 ml. per minute at this stage caused decrease of density. Increase of growth rate after aeration necessitated adjustment of influx at 15 minute intervals until it reached a maximum; the necessary dilution rate then reached was about 25 ml. of medium per minute when oxygen tension was low. Bacterial concentration was maintained between 0·15 mg. per ml. and 0·30 mg. per ml., which corresponded to that at the beginning of the logarithmic phase in culture not submitted to continuous dilution. pH remained between 6·6 and 6·9. 2 ml. samples were removed at intervals for manometry. The CO_2-buffered cups contained 3 ml. of 4 M diethanolamine previously equilibrated to 5 p.c. CO_2. These were gassed from a bye-pass from the gas used in aeration. The manometric measurements in air and 5 p.c. CO_2 were each in triplicate.

Anaerobic conditions were obtained for a control experiment during continuous dilution by aerating with oxygen-free nitrogen, prepared by passing nitrogen over a long column of yellow phosphorus pellets, followed by washing in water.

Examination of cytochrome a_2 during aeration.

In a separate experiment samples of paste were prepared from cultures grown at high and at low oxygen tension. Cytochrome a_2 was estimated by

matching the intensity of the absorption band of *E. coli* at 640 mμ against the 630 mμ band of a standard solution of hemiglobin contained in a wedge trough as described by Keilin and Hartree (1946).

Five litres of culture grown by the continuous culture method were aerated very vigorously by gas mixtures which produced oxygen tensions of approximately 0·75 atm. and 0·1 atm. respectively.

Two litres of culture were withdrawn at half-hourly intervals and centrifuged. The paste so obtained was placed under the microscope objective in a small wedge. The optimum depth of the paste for observation of the cytochrome a_2 band was 0·6 cm. This was maintained in all experiments, as was the optimal adjustment of the slit of the spectroscope, and the intensity of the light source. Adjustment of light through the standard matching solution was arranged so that background illumination was equal in standard and paste. Bacterial density of the paste was estimated from its protein content using the biuret method of Strickland (1951).

An attempt was made to estimate the amount of cytochrome b_1 present, by matching its band at 560 mμ against that of reduced cytochrome c at 550 mμ. However, optimal conditions for a_2 observations allowed poor distinction of the intensity of the b_1 band. The band of cytochrome a_1 at 590 mμ was too faint to attempt measurement.

RESULTS.

Respiration.

Table 1 shows the Q_{O_2} of *E. coli* grown for 4½ hours in continuous culture at oxygen tensions between 0·09 and 0·95 atm. These results were taken from manometry in 5 p.c. CO_2. It was found that the Q_{O_2} of non-aerated cells at zero hours was slightly less in the 5 p.c. CO_2 buffered series when oxygen tension was high than in the CO_2-free air atmosphere of Warburg cups. The low Q_{O_2} of 540 and 520 at zero hours in two experiments may be due to the fact that in these experiments aeration was commenced somewhat earlier after adding the culture to the aerator than in the others. The highest values of Q_{O_2} reached between 3 and 4½ hours were from 910 to 1,090.

The fact that increase of Q_{O_2} is not due to factors dependent on continuous culture is shown by the constant Q_{O_2} obtained in the anaerobic series.

TABLE 1.

Increase of Q_{O_2} during aeration.

P_{O_2}	Hours of aeration			
	0	1½	3	4½
0·90–0·95	670	960	1090	950
0·75–0·87	540	890	1000	940
0·42–0·45	640	—	970	980
0·1–0·3	520	830	910	1020
Anaerobic	650	650	680	—

Growth rate.

It was noticed during continuous culture that the rate of influx required for constant density was greatest when p_{O_2} was low. It was noticed during manometry also that the growth was higher when p_{O_2} was low. Table 2 shows the value of k in terms of specific growth rate per hour. These values were obtained during manometry. Growth of culture grown at oxygen tension 0·1 to 0·3 atm. was more rapid than that at 0·9–0·95 atm., and in the anaerobic culture. The Q_{O_2} of the latter was measured in 5 p.c. CO_2 and 95 p.c. O_2.

TABLE 2.

Specific growth rate per hour (k) during growth at different oxygen tension.

P_{O_2} of culture	P_{O_2} in Warburg flask	Hours of aeration 0	$1\frac{1}{2}$	3	$4\frac{1}{2}$
Anaerobic	0·95	0·53	0·55	0·37	0·4
0·1–0·3	0·1–0·3	0·80	0·84	1·44	1·56
0·90–0·95	0·90–0·95	0·35	0·82	0·86	1·17

Variation of cytochrome a_2 concentration with oxygen tension.

Table 3 shows the amount of cytochrome a_2 produced in two experiments during aeration for $4\frac{1}{2}$ hours, when oxygen tension was held at about 0·1 atm. and 0·75 atm. respectively.

TABLE 3.

Development of cytochrome a_2 during aeration.

P_{O_2}	Hours of aeration 0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3	$3\frac{1}{2}$	4	$4\frac{1}{2}$
0·1	0	0	0	10	—	20	—	22	32	32
0·75	0	8	8	16	16	16	16	16	16	16

The units of measurement used are mm. of a layer of haemiglobin solution being $4{\cdot}85 \times 10^{-5}$ molar for haem, which matched the a_2 absorption band of a standard cell paste of 0·6 cm. thickness containing 100 mg. per ml. dry weight of bacteria. Each is the result of at least four readings.

It is seen that the a_2 band developed more rapidly at 0·75 atm. than at 0·1 atm. Absorption, equivalent to 8 mm., is reached in 30 minutes at 0·75 atm., whereas there is no a_2 detectable in the 0·1 atm. sample in 60 minutes. After 60 minutes, however, formation of a_2 became more rapid in the 0·1 atm. culture. At $4\frac{1}{2}$ hours the 0·1 atm. culture contained twice as much a_2 as the 0·75 atm. sample.

It was said earlier that the effluent from the continuous culture used for measurements of Q_{O_2} was examined for the presence of cytochromes. It was noticed repeatedly that the a_2 content was high when oxygen tension was low and that the band at 630 mμ could often not be seen when p_{O_2} approached saturation. Addition of broth to the medium appeared to increase a_2 formation.

The interval required for preparation of cell paste allowed ample time for reduction of cytochromes. It was found on many occasions that addition of dithionite or incubation with glucose failed to increase the strength of the a_2 band, when it was found to be poorly developed, after vigorous aeration. The a_2 band from cells produced at high oxygen tension was therefore produced by cytochrome a_2 in the fully reduced state. Cytochrome b_1 which was absent in anaerobic cells produced an intense band at 560 mμ, within 4 hours, on aeration.

DISCUSSION.

Table 4 shows the ratio of Q_{O_2}/a_2 during 4½ hours of aeration at low and high p_{O_2}. At low p_{O_2} the cytochrome a_2 content increased much more than Q_{O_2} between 1½ and 4½ hours, while at high p_{O_2} the Q_{O_2}/a_2 ratio remains steady. It is evident that the cytochrome a_2 content and the corresponding Q_{O_2} are not closely related, although both rise during aeration. It is possible that excess cytochrome a_2 may be formed at low p_{O_2}, and that this may be counterbalanced at high p_{O_2} by a destruction of cytochrome a_2.

TABLE 4.

Q_{O_2}/cytochrome a_2 units.

P_{O_2}	Hours 1½	3	4½
Low[1]	83	45·5	34
High[2]	60	68	59

[1] Q_{O_2} and cytochrome a_2 units at a p_{O_2} of about 0·1.
[2] Q_{O_2} at a p_{O_2} of about 0·9, cytochrome a_2 units at a p_{O_2} of about 0·75.

The higher growth rates of cells grown at low p_{O_2} shown in Table 3, which suggests at least superficially a relationship between a_2 content and growth rate, may also be explained by O_2 poisoning.

Another possibility is that cytochrome a_2 is concerned in directing aerobic energy towards cell synthesis and that cytochrome a_1 functions as the real oxidase.

SUMMARY.

A continuous culture apparatus is described which permits growth while maintaining bacterial concentration and oxygen tension within narrow limits.

The mathematical theory of the method is discussed.

A strain of *E. coli* was found to develop cytochromes a_2, a_1, and b_1 on aeration.

The Q_{O_2} of aerated *E. coli* was found to increase as the result of aeration. The Q_{O_2} reached was not closely related to the amount of cytochrome a_2 present.

Possibilities concerning the function of cytochrome a_2 are discussed.

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ACCELERATION AND RETARDATION OF THE PROCESS OF AXON-SPROUTING IN PARTIALLY DENERVATED MUSCLES[1]

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In previous papers the process whereby denervated muscle-fibres, in partially denervated muscles, are re-innervated by collateral and ultraterminal sprouting of surviving intact nerve-fibres, has been extensively described (Edds, 1950; Hoffman, 1950). The mechanism has been analysed to some extent in regard to the operation of peripheral factors (Hoffman, 1950; Hoffman and Springell, 1951): it is suggested that, following breakdown of the nerve-fibre, a moderately unsaturated fatty acid (in glyceride form) is released and diffuses into the tissue spaces. This substance (known as "neurocletin") is envisaged as penetrating neighbouring axons at specific points, causing breakdown of the axolemma (limiting membrane of the axon) and liquefaction of the cortical axogel, followed by outflow of axosol and initiation of sprouting.

Following the development of this hypothesis regarding the operation of peripheral factors in sprouting, it was felt that further information concerning both central and peripheral factors might be obtained by the detection of accelerators and inhibitors of sprouting in partially denervated muscles. With reference to central regulating mechanisms, Weiss (1941) and Weddell and Zander (1951) in discussing similar axon-sprouting in cutaneous nerve plexuses, have suggested that turgor or pressure of axoplasm within the neurone, is the crucial factor in sprouting. According to these authors the nerve-ending expands automatically into territory rendered unoccupied by the degeneration of a neighbouring ending, this expansion resulting from the raised internal pressure of axoplasm. If this cellular turgor is maintained by the synthesis of new axoplasm in the cell body (Caspersson, 1947; Hyden, 1943; Landstrom, Caspersson and Wohlfart, 1941), modification of this synthetic activity should result in changes in cell turgor.

Changes in the cell body, during regeneration of the axon, have long been known: Bodian and Mellors (1945) have analysed the reaction into a regressive

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phase, corresponding to the period of chromatolysis, followed by a regenerative phase (which will in future be termed "chromatogenesis") corresponding to the restoration of the Nissl substance. They showed that the chromatolytic phase represented a period of intensive enzymatic activity, and considered that it represented the synthetic effort of the cell, preliminary to the replacement of the axon. One of the purposes of the experiments described below was to attempt to modify the course of axon-sprouting by inducing comparable changes in the synthetic activity of the neurone. The means chosen have been electrical stimulation of the neurone, and administration of the dye Pyronin.

A number of observations on changes in Nissl granules, following electrical stimulation of neurones, may be found in the earlier literature, but one of the first systematic studies was that of Hyden (1943). This worker stimulated dorsal spinal nerve roots with sinusoidal alternating current of 1-2 mA for 1-10 minutes, and studied the changes occurring in the cells of the ganglia. The reaction varied according to the duration of treatment, but in general a substantial increase in the amount of Nissl substance was observed, and extensive changes in the nucleolus were also detected. Barr and Bertram (1949) reported depletion of Nissl substance following electrical stimulation, and Sulkin and Kuntz (1950), after stimulating visceral ganglia, described extensive histological and histochemical changes in the neurones leading them to suggest that synthetic activity had been heightened. Liu-Chan, Bailey and Windle (1950) claimed that the only changes detectable in electrically stimulated neurones resulted from accidental damage to the axons during manipulation; however, a more extensive study by Barr and Bertram (1951) has led to the most complete description, at present available, of the changes occurring in the neurone after electrical stimulation. During the first three days after stimulation, chromatolysis, nucleolar enlargement and migration of the nucleolar satellite to the interior of the nucleus, are observed. (The nucleolar satellite is a clump of Feulgen-positive chromatin, normally found close to the nucleolus in cat neurones.) Later these processes are reversed, resulting in shrinkage of the nucleolus, return of the satellite, and chromatogenesis. This latter process results in a rapid rise in concentration of ribonucleotides, which eventually attains a level much higher than normal, then slowly returns to normal. These results appeared to indicate that the cytoplasmic synthetic system of the neurone is markedly affected by electrical stimulation, and seemed to provide a promising method for increasing cellular turgor, and hence, possibly, for altering the rate of sprouting.

Some observations of Speidel (1941) seemed to suggest a second line of approach to the problem of axon-sprouting. This author observed, *in vivo*, that the mitosis of Schwann and other cells had a profound effect on resting nerve-endings, and, further, described the mitosis of a Schwann cell as a necessary preliminary to node formation on the axon, and the outgrowth of a collateral branch. Edds (1950) postulated that the proliferation of Schwann cells, follow-

ing Wallerian degeneration of the nerve-fibres on which they lay, might be the source of the stimulus to sprouting of intact axons. Mitosis of Schwann cells, as a mode of formation of new nodes, might also prove to be significant in regard to the localization of sprouting: it is a conspicuous feature of this sprouting that one rarely encounters sprouts other than at nodes, so that sprouting occurs only at established nodes, or else the stimulus induces Schwann cell mitosis and node formation before the outgrowth of the new sprout. It has, in fact, been demonstrated (Weiss, 1950b; Overton, 1951) that nervous tissue contains a mitogenetic substance not found in other tissues. The correlation of Schwann cell activity and axon sprouting was further supported by the observations of Rexed and Rexed (1951) who reported that, in re-innervation of the partially denervated cornea, Schwann cells showed neither proliferation nor hypertrophy, and intact axons near the denervated zone failed to sprout collaterals. They attributed the absence of Schwann cell reaction to the presence in the ground substance of the cornea of high concentrations of muco-polysaccharides, which are known to be antimitotic (Heilbrunn and Wilson, 1949; Balasz and Holmgren, 1949; Harding, 1949a, b).

These latter observations appeared to provide a method of determining the rôle of Schwann cell mitosis in the initiation of sprouting. A number of mitotic inhibitors were therefore tested for their effects on sprouting in partially denervated muscles.

Experimental.

Except in the experiments with orally-administered dyes, and the second series with cryptopleurine, albino rats from an inbred Wistar line have been used; wherever possible batches have consisted of litter-mates. Partial deneurotisation of the sciatic plexus has been achieved by section of the fifth lumbar nerve (L5), and after appropriate times the animals were killed and the muscles—gastrocnemius and soleus—dissected free and prepared for examination by the Loewit gold method.

All drugs (except dyes and cryptopleurine) were administered intraperitoneally; the dyes were administered orally, while cryptopleurine was injected subcutaneously.

Quantitative methods.

The most important requirement in the investigation of rate of sprouting is an adequate method for determining extent of re-innervation. Edds (1950) used a ratio which he called "terminal innervation ratio"; this consisted of the number of end-plates innervated by a terminal nerve-bundle divided by the number of nerve-fibres entering this bundle. This ratio he found to average 1·1 in the normal rat serratus anterior muscle, while after sprouting had occurred it might reach an average of 3·0–4·7, depending on the original degree of denervation. Whilst this method gives a convincing statistical demonstration that axon-sprouting has occurred, it does not permit any determination of extent of re-innervation, since the ratio must vary with the extent of denervation, and cannot reach any absolute limit.

A more adequate measure of extent of re-innervation has been developed for use in the experiments described below; it involves counting the number of residual nerve-fibres ("R"), denervated endoneurial tubes ("D") and new sprouts ("S"). The sprouts are detected

by their small diameter, lack of myelin and nodes, and their association with hypertrophied Schwann cells: occasionally they end blindly within the endoneurial tubes. The hypertrophied Schwann cells also serve often as a guide to the endoneurial tubes still denervated.

The ratio used below (''re-innervation percentage'') reflects the proportion of *endoneurial tubes* re-innervated at any given stage, and is obtained from $\frac{S \times 100}{S + D}$. This provides an absolute basis for comparison, since it rises from 0–100 as far as can be detected. Histologically all endoneurial tubes appear to be re-innervated, except when either re-innervation is accomplished by ultraterminal sprouting, or, rarely, when collaterals fail to utilize endoneurial channels.

In the Tables to be found under each experimental heading, ''percentage of residual innervation'' is tabulated alongside ''re-innervation percentage''. Residual innervation is obtained from the ratio $\frac{R \times 100}{R + S + D}$ and the mean for the 117 animals for which this has been determined (this excludes those from which data in Table 8 were obtained) is 48·6 p.c. This indicates that L5 section denervates these particular muscles approximately by half.

Statistical analysis has been carried out by conversion of the data (in percentages) to normal distribution by means of Fisher's inverse sine transformation (Fisher and Yates, 1943). Means and 95 p.c. confidence limits have been computed, and reconverted to percentages. Significance has been assessed by the ''T'' test, and is stated at the bottom of each Table in terms of odds against experimental and control results originating from a single, randomly varying, population.

Errors which tend to complicate the determination of these ratios have been encountered. The first, concerning failure of a proportion of sprouts to utilize endoneurial channels, has already been mentioned: a second source arises from unsatisfactory staining of endoneurial tubes, while a third factor arises as a result of maturation of the sprouts. Even in the control series, thickening and myelination of sprouts commences after about seven days, and this interferes in their distinction from residual fibres.

In all but the dye-fed and second cryptopleurine series, between one and two hundred originally-denervated endoneurial tubes have been counted: in the two series excepted above, approximately two hundred were counted in each case. Although commonly many more could be counted, this has been found to be an effective upper limit to counts, where a large number of animals is used.

Control series.

Quantitative data have been obtained in twenty-one animals in this series, divided into groups killed 4, 7 and 14 days after operation, while information concerning the appearance of material three days after operation has been obtained from earlier investigations. The extent of sprouting at various times after operation within this series is set out in Table 1.

Observations. On the first and second days after operation only degenerative changes in the interrupted fibres are observable: they fragment, forming large, darkly staining ''ovoids'' which may also be seen within the fragmenting end-plate, and which are gradually removed, partly by macrophagic engulfment. On the third day the first sprouts are seen, invading the denervated tubes, which by this time are largely clear of ovoids and debris (although these persist longer within the sole-plate) and are now occupied by characteristic Büngner-bends—swollen columns of Schwann cells with large, pale nuclei (see Figs. 1 and 2). At this stage the percentage of tubes entered by sprouts is too small to be estimated quantitatively.

TABLE 1.

Re-innervation percentages in control series.

4 days			7 days			14 days		
Animal	Re-inn. p.c.	Residual	Animal	Re-inn. p.c.	Residual	Animal	Re-inn. p.c.	Residual
Con. 1	9·0	48·4	Con. 5	17·9	59·3	Con. 10	47·7	41·4
Con. 2	8·6	64·2	Con. 6	26·0	62·0	Con. 11	34·6	69·1
Con. 3	2·9	52·1	Con. 7	18·0	50·5	Con. 12	43·2	60·8
Con. 4	5·1	50·4	Con. 8	30·0	51·4	Con. 13	52·5	46·4
Con. 14	15·8	67·1	Con. 9	18·8	53·3			
Con. 15	16·4	58·5	Con. 18	23·6	38·2			
Con. 16	11·3	41·1	Con. 19	20·3	31·2			
Con. 17	4·2	38·5	Con. 20	26·0	35·0			
			Con. 21	22·7	46·3			
Means and confidence limits		13·4			26·2			53·5
		8·5			*22·4*			*44·5*
		4·8			19·4			35·5

By the fourth day sprouts are far more numerous, as can be seen from Table 1, and the mean re-innervation percentage has risen to 8·5: most of the debris has, at this stage, disappeared from the tubes. By the seventh day this percentage has reached 22·4, and by this time few terminal bundles which have suffered denervation are without at least one sprout. Meanwhile the sprouts are thickening, and maturation is commencing. After fourteen days, re-innervation has reached 44·5 p.c. and maturation is well advanced, some sprouts being already myelinated. Figs. 6 and 7 illustrate the appearance of sprouts at this stage.

The incidence of sprouting varies somewhat from one terminal bundle to another, so that some residual fibres may each possess several collaterals, whilst others may have none. This observation, together with the indication that an abrupt onrush of sprouting occurs during the fourth day after operation, is discussed later in terms of responsiveness of the neurone to neurocletic stimulation.

Electrically stimulated series.

Two groups of experiments are included under this heading; in all, results obtained from seventeen animals are tabulated.

(a) *E* series (electrical stimulation of the spinal cord). Immediately following section of L5, laminectomy was performed in the region of L3 and L4, and flattened silver electrodes inserted over the spinal cord. Later this procedure was modified, two small holes being drilled in the neural arch over L3 and fine, perspex-insulated, silver wires inserted to rest on the cord. With both methods of application, fine, floating wire leads were found to be essential owing to the convulsions which followed application of the current. Sinusoidal alternating current was used, obtained in early experiments from a 6·3 volt filament transformer in series with a 0-5,000 ohm variable resistance and a rectified moving-coil milliammeter. Attempts to utilize square-wave direct current from a constant-voltage stimulator proved

unsatisfactory—polarization reduced the current to a negligible amount within ten seconds. In later experiments current was supplied from a beat-frequency oscillator, permitting frequencies of 1-17,000 to be used.

The animals were stimulated, usually at 50-100 cycles, with a current of 1·5 mA, adjusted for each animal by means of the variable resistance, for periods of ten minutes to one hour. As soon as the current was applied both hind limbs stiffened in tetanic extension, which persisted for two to three minutes, then gradually decreased as the muscles fatigued. Throughout the period of stimulation little variation in current was observed. In a few cases insulated silver electrodes were implanted over the spinal cord, and current passed daily, after operation, for ten minutes. This was discontinued as it seemed to produce no increased response.

(b) *NE* series (electrical stimulation of the lumbosacral plexus). In this group of three animals, the sciatic roots were stimulated, immediately after section of L5, with perspex-insulated silver electrodes. Under these conditions only the operated limb extended tetanically on application of current. Details of current, frequency and duration were as in the *E* series.

TABLE 2.

Re-innervation percentages in electrically stimulated series.

4 days			7 days		
Animal	Re-innervation p.c.	Residual	Animal	Re-innervation p.c.	Residual
E1	23·6	50·5	E21	31·4	56·9
E7	17·1	42·0	E22	36·3	52·8
E8	25·2	35·6	E23	29·8	57·1
E12	24·5	63·6	E24	30·9	64·8
E14	14·8	54·0			
E15	33·2	47·3			
E16	24·7	54·2			
E17	21·9	57·0			
E18	19·3	55·1			
E20	28·9	54·6			
Means and confidence limits	27·8 / *23·5* / 20·8 (control mean 8·5)			36·7 / *32·2* / 27·5 (control mean 22·4)	
Odds favouring a significant increase over control.	50,000 : 1			500 : 1	
NE1	31·0	52·1			
NE2	44·3	62·3			
NE7	22·6	43·6			
Means and confidence limits	61·1 / *32·3* / 9·5				

Observations. Examination of the preparations from animals in these two series immediately indicated a substantial increase in sprouting, as may be seen from the figures in Table 2. Re-innervation has reached the level of 23·5 p.c. four days after operation, in the *E* series, in contrast to the comparable control figure of 8·5 p.c. After seven days, the mean re-innervation percentage is 32·2,

DESCRIPTION OF PLATES.

PLATE I.

Fig. 1. Bands of Büngner (arrows) seen in a partially deneurotized nerve-bundle, four days after operation. Control material; gold × 750.

Fig. 2. Büngner bands in another partially deneurotized bundle, from control material, four days after operation. Note that, in both of these figures, the cells are swollen, and there is no stainable debris. Gold × 750.

Fig. 3. Persistence of gold-staining ovoid debris (arrows) along deneurotized endoneurial tubes, in an animal treated for seven days after operation with heparin. Gold × 550.

Fig. 4. In the denervated tube running horizontally, right, across the field further ovoid debris is clearly seen. Four days after operation, from an animal treated with sodium alginate. Gold × 600.

Fig. 5. Persistence of ovoids both along the endoneurial tubes and within the denervated sole (*s*) in an animal treated for seven days after operation with heparin.

PLATE II.

Fig. 6. Single sprout runs alongside two residual fibres in a terminal bundle: note that the sprout is fairly thick and substantial. From control material, fourteen days after operation. Gold × 500.

Fig. 7. Within a terminal bundle a single thick sprout may be seen clearly, while parts of several others are in focus. From control material, fourteen days after operation. Gold × 600.

Fig. 8. Within a terminal nerve-bundle may be seen one residual nerve-fibre (*r*) and at least four sprouts (*s*). From an animal which, four days earlier, received electrical stimulation of the spinal cord (lumbar) immediately after L5 section. Gold × 400.

Fig. 9. In this figure, a residual nerve-fibre (*r*) has sprouted at a node (*n*) and the new branch has reneurotized one of a group of Büngner bands (*b*), passing upwards in the field and branching, eventually reconstituting two denervated end-plates. Four days after operation and electrical stimulation. Gold × 550.

Fig. 10. Within the terminal bundle illustrated here, three residual fibres and four sprouts may be detected. Four days after operation and electrical stimulation. Gold × 400.

Fig. 11. In this figure is illustrated the occurrence of an ultraterminal end-plate (*ul*) and a collateral sprout (*col*) within a single terminal bundle. Four days after operation and electrical stimulation. Gold × 400.

PLATE III.

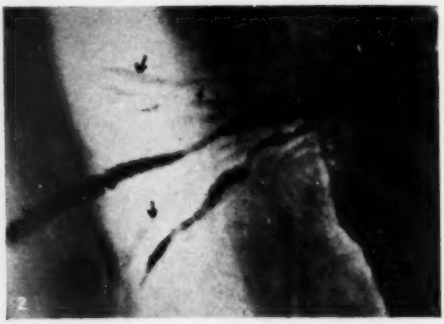
Fig. 12. From a terminal bundle emerges a single residual fibre (*r*), together with numerous sprouts, some bearing irregular varicosities. From an animal treated with colchicine for seven days after operation (Co 5). Gold × 400.

Fig. 13. Within a single large nerve bundle, four sprouts (*s*) may be seen. This specimen is from an animal treated with pyronin for fours days after operation. Gold × 450.

Fig. 14. This is a slightly higher-resolved view of part of the field illustrated in Fig. 12. In this figure, the varicosity (*v.r.*) at the point on the residual fibre whence a sprout originates, is clearly seen. A peculiar, ring-shaped varicosity (*v.s.*) is also seen on another sprout. Gold × 500.

Fig. 15. This photograph is from another specimen obtained from the same animal as Figs. 12 and 14. Note the numerous sprouts within the terminal bundle. Gold × 400.

Fig. 16. This figure illustrates the multiple sprouting at a single point (*l.*) encountered in atypical response to colchicine; the arrow points to the swollen and distorted region of the residual nerve-fibre from which four sprouts emerge. One of these sprouts passes out of the plane of focus to branch in turn, forming two further sprouts. These new branches are not all, as the photograph would suggest, blind terminals—their terminal portions are, however, completely out of focus. From another preparation from animal Co 5. Gold × 650.



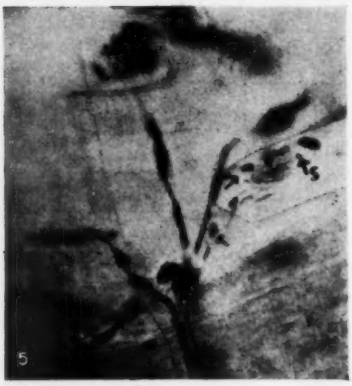
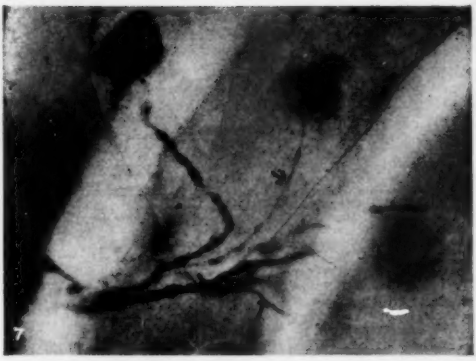


Plate I.



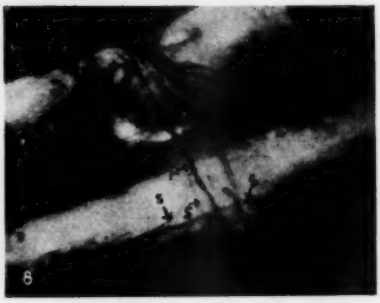


Plate II.

Plate III.

contrasting with that for the control group of 22·4. Four animals examined three days after operation and electrical stimulation yielded re-innervation percentages comparable with those for the control three-day group: however, since these are too small to have quantitative validity, they have not been tabulated. In a comparison of re-innervation percentage four days after operation with electrical stimulation (mean 23·5) with that for the control group seven days after operation, analysis resulted in odds 5:1 in support of the conclusion that the difference is within limits of random variation. Since the bulk of the sprouting encountered after four days occurs during the fourth day, this means that as much sprouting occurs in one day, if electrically stimulated, as occurs during four days in the controls.

The histological appearance of the material fully bears out these quantitative results: the effect is apparently an overall one, sprouts appearing in practically all the terminal nerve-bundles after four days, and individual sprouts are thicker than their control homologues. In Plate 2, comparison may be made between the appearance of the control material fourteen days after operation (Figs. 6 and 7) and that of sprouts four days after operation with electrical stimulation (Figs. 8-11). In some cases more than one sprout is seen in a single terminal nerve-bundle; a case of this kind is illustrated in Fig. 11, where a collateral lies close to an ultraterminal. In Figs. 8 and 10, several collaterals may be seen in each terminal bundle. Maturation is detectable in some of these sprouts, and several obvious collateral and ultraterminal sprouts were actually myelinated and possessed nodes. This indicates rapid growth and development, since these fibres are almost certainly no more than twenty-four hours old.

The appearance of sprouts in a higher proportion of terminal nerve-bundles, after electrical stimulation, suggests that more neurones have been rendered responsive to the peripheral stimulus, possibly by increase in the metabolic activity of the perikaryon. The process was not followed beyond seven days, in view of the interference with identification, caused by maturation.

In the *NE* series, in which the intact roots of the sciatic plexus were stimulated, figures were obtained which, although too few to provide a complete analysis, indicated as great, if not greater, increase in rate, when compared with the *E* series. The point of stimulation of the nerves was too far from the cord (approximately one centimetre) to permit current spread to the cells, and selective stimulation of the right limb muscles (confirmed by observation) supported the assumption. It may therefore be concluded that the effect was exerted by antidromic nerve-impulses bombarding the cell bodies.

Effect of frequency variation. Although no systematic investigation has been made into the effect of varying frequency of stimulation, the few observations made tend to indicate that frequencies up to 17,000 cycles still produce the acceleration. Stimulation at twenty-four hour intervals, by means of buried electrodes, did not produce any marked differences. Thus, as far as can be determined, the changes in the cells resulting in the acceleration of sprouting

only commence after the third day, and further stimulation between the time of operation and this onset cannot accelerate it. This delay corresponds with the period of chromatolytic change described by Barr and Bertram (1951), so that it appears that the chromatogenic and other restorative phenomena are temporally correlated with the acceleration of sprouting, as they are with the advance of the axon in nervous regeneration (Bodian and Mellors, 1945).

Dye-fed series.

(a) *Pyronin.* Following the report of Clark and Gunson (1953) that the dye Pyronin, when fed to, or injected into Drosophila, has proved markedly mutagenic and has also consistently accelerated growth rate, and since Pyronin is known to form a relatively stable compound with low-polymerized nucleic acid (such as the ribo-nucleic acid of the Nissl substance) it was decided to test its effect on axon sprouting.

In all, twenty rats from a mixed population of hybrid rats (progeny of a Hooded × Albino cross) were given a 0·1 p.c. solution of Pyronin as drinking water from four days before operation, then thereafter until killed. After about two days, exposed areas of skin assumed a rosy tint, and dye was excreted in urine. Deneurotization was accomplished, as before, by L5 section, and the animals were killed in groups 3, 4, 7 and 14 days after operation. Since the rats used in this series differed genetically from those used previously, a separate batch was used to provide control groups killed 4 and 7 days after operation. In this experiment, counts were all taken as near as possible to 200 originally-denervated tubes (S + D). Both types of Pyronin have been tested: Pyin. 1 and 2 received Pyronin B (ethyl homologue—Merck), Pyin. 10a and 10b received Pyronin G (or Y, methyl homologue, Gurr), while the rest received a mixture of both samples.

Observations. As may be seen from Table 3A, sprouting on the third day was still extremely slight, and it was not considered necessary to prepare a control group for this stage: as may be seen, sprouts range from 0–3·5 p.c. On the fourth day, however, an extremely marked increase became evident, and the experimental figures in Table 3A (41·7 p.c. after four days) may be contrasted with the control figure in Table 3B of 5·8 p.c. re-innervation. Similarly, after seven days, an experimental re-innervation of 80·8 p.c. contrasts clearly with the control figure of 24·7 p.c. After these seven-day figures had been obtained, it was not considered necessary to count the fourteen-day Pyronin material.

Histologically the effect becomes immediately obvious and may be inferred from an examination of Fig. 13, where four sprouts may be seen within a single nerve-branch, which has suffered only moderate denervation, four days after operation. Several sprouts may be seen within almost every denervated bundle four days after operation, and in some branches every denervated tube has been invaded. Sprouting is, in fact, so rapid that many sole-plates become re-inner-

vated before the ovoid debris has been removed. After seven days, denervated tubes are relatively uncommon, but they may still be detected even after fourteen days; presumably the process slows down after the initial burst. Maturation seems to parallel sprouting, as was found in the electrically-stimulated animals, and some of the short ultraterminal processes appeared mature and myelinated.

TABLE 3.

Re-innervation percentages in dye-fed series.

	(A) Controls			(B) Pyronin-fed			Other dyes	
	Animal	Re-inn. p.c.	Residual	Animal	Re-inn. p.c.	Residual	Animal	Re-inn. p.c.	Residual
3 Day				Pyin. 3	3·0	31·7			
				Pyin. 4	0	25·2			
				Pyin. 5	3·0	34·9			
				Pyin. 6	3·5	36·4			
								(C) Rhodamine	
4 Day	Con. 1	6·0	36·5	Pyin. 1	45·0	48·1	ROD 1	3·0	43·3
	Con. 2	7·2	60·3	Pyin. 2	30·5	52·8	ROD 2	2·0	38·2
	Con. 3	2·0	47·1	Pyin. 7	46·0	47·6	ROD 3	1·5	49·5
								(D) Methylene blue	
	Con. 4	4·0	57·0	Pyin. 8	41·0	45·4	MEB 1	6·9	63·2
	Con. 5	7·3	51·8	Pyin. 9	44·8	55·9	MEB 2	1·0	55·0
	Con. 6	10·5	59·2	Pyin. 10	43·0	66·0			
Most likely value, and C.L.'s		9·4 *5·8* 3·0			47·8 *41·7* 35·5			5·7 *2·6* 0·8	(combined other dyes)
Odds favouring significant increase					greater than 1,000: 1				
7 Day	Con. 7	22·3	58·4	Pyin. 10a	78·7	62·4			
	Con. 8	19·0	50·5	Pyin. 10b	76·0	56·4			
	Con. 9	28·0	36·6	Pyin. 11	78·8	64·1			
	Con. 10	25·3	30·3	Pyin. 12	87·2[1]	34·3			
	Con. 11	23·2	40·0	Pyin. 13	79·2	62·5			
	Con. 12	31·5	54·8	Pyin. 14	83·6	47·9			
Most likely values and C.L.'s		32·9 *24·7* 20·3			85·0 *80·8* 76·2				
Odds favouring significant increase					greater than 1,000: 1				

[1] Only 100 fibres counted, owing to inadequate material.

(b) *Other dyes.* In order to determine the specificity of this effect, two other dyes, closely related to Pyronin, were next tested. The first, rhodamine, differs from Pyronin in that a further benzene ring (with a carboxyl group in the *ortho* position) is attached to the central carbon atom; the second, methylene blue, differs from Pyronin in the form of the central ring. Pyronin is xanthene in type, methylene blue thiazine in type. Both of these dyes were administered in drinking water, in the same concentration as was Pyronin, and the animals

operated and killed four days later. The results of counts are set out in Table 3C and 3D, and although too limited to assess in terms of the controls, their difference from those obtained for Pyronin is obvious. In each case, urinary excretion and skin colouration indicated effective penetration of the dye to the tissues.

These results tend to indicate that the effect is due to the specific molecular configuration of Pyronin: no difference in response between those animals receiving the ethyl- and methyl-homologues has been observed, however, although insufficient data are available to permit detection of other than very marked differences in potency. Clark and Gunson report that ethyl-Pyronin (Pyronin B) is far more mutagenic than the methyl form.

The extent of acceleration far exceeds that produced by electrical stimulation but tends to parallel it in that the process does not accelerate until the fourth day—in this case re-innervation rises from 3 p.c. to over 40 p.c. in twenty-four hours.

Antimitotic substances.

In this group of experiments, as well as determining sprouting rate by counting denervated and re-innervated tubes, the effect of the treatments on Schwann cell activation and mitosis has been determined by examination of the partially deneurotized nerve trunks. The nerves were fixed in 10 p.c. neutral formalin, sectioned at 8-9μ and stained with a modified Giemsa technique, which yielded excellent detail of Schwann cells, axonal and myelin debris, and macrophages and other phagocytic cells.

(a) *Cytoplasmic inhibitors.*

(i) *Muco-polysaccharides.* In view of the suggestions of Rexed and Rexed (1951) it was considered desirable first to apply drugs from within this group. A number of these is known to possess anti-mitotic properties (Harding, 1951a) including heparin (Heilbrunn and Wilson, 1949; Harding, 1949; Balasz and Holmgren, 1949) sodium agar (Balasz and Holmgren, 1949) and Shear's polysaccharide (Harding, 1951b). Two examples were chosen for application to partially denervated muscles: heparin (Boots) and sodium galinate. This latter is closely related to agar, and obtained from similar sources (marine algae). Further, it is obtainable commercially.[3]

Heparin. This drug was administered at the rate of 10-20 mg./kg. once or twice daily, from immediately after operation. It was found to produce extensive haemorrhage and the abdominal region soon showed extensive haematomas, resulting from the intraperitoneal mode of administration. As a result, mortality was high, and it was difficult to obtain sufficient material for study.

Observations. Only six of the treated animals survived for the required times, two each being killed after four, five and seven days. However, marked

[3] "Manucol" (I.C.I.). I wish to thank I.C.I.A.N.Z. for the kind provision of a sample.

changes were observed in the histological material, and in extent of sprouting. The normal absorption of gold-staining debris from the degenerating nerve-fibres was significantly retarded; while endoneurial tubes are usually clear of gold-staining ovoids and the Schwann cells enlarged after the third day (see Figs. 1 and 2) the material from the heparin-treated animals showed much greater persistence of ovoids, while Schwann cell hypertrophy was substantially reduced. Figs. 3 and 5, representing seven-day heparin-treated material, clearly show this persistence of ovoids (arrows) within the denervated tubes.

TABLE 4.

Re-innervation percentages in muco-polysaccharide treated series.

4 days			5 days			7 days			14 days		
An.	Re-inn. p.c.	Resid.	An.	Re-inn. p.c.	Resid.	An.	Re-inn. p.c.	Resid.	An.	Re-inn. p.c.	Resid.
(A) Sodium alginate											
Alg. 3	1·3	35·6				Alg. 7	15·9	38·8	Alg. 9	27·9	53·1
Alg. 4	1·3	57·4				Alg. 8	6·5	49·3	Alg. 10	30·5	53·8
Alg. 5	1·2	41·7				Alg. 14	7·1	52·7			
Alg. 6	4·1	60·0				Alg. 15	5·4	57·1			
						Alg. 16	6·2	54·5			
						Alg. 17	7·5	40·2			
						Alg. 18	15·7	46·0			
						Alg. 19	5·0	47·7			
						Alg. 20	4·5	53·3			
						Alg. 21	7·0	38·4			
Means and C.L.'s		4·2						10·5			47·2
		1·8						*7·8*			*29·1*
		0·5						5·2			13·9
		(control mean 8·5)						(control mean 22·4)			(control mean 44·5)
Odds favouring significant reduction: 260: 1						Greater than 100,000: 1					
(B) Heparin											
Hep. 4	0	47·7	Hep. 2	3·8	54·3	Hep. 6	2·0	55·6			
Hep. 5	1·8	44·3	Hep. 3	1·3	23·0	Hep. 7	3·3	47·1			
Means	*0·5* (con. 8·5)			2·3			*2·7* (con. 22·4)				
Odds favouring reduction	160: 1					greater than 100,000: 1					

Sprouting was markedly reduced, as may be seen by reference to Table 4B. After the fourth day the most sprouting encountered was 1·8 p.c., while after seven days a maximum of 3·3 p.c. was obtained.

The denervated tubes in the nerve trunks showed a decreased activation of Schwann cells, few mitotic figures, decreased absorption of debris with no recognizable macrophages.

Sodium alginate. The powder was dissolved in isotonic saline to form a 1 p.c. solution of moderate viscosity which was then Seitz filtered and injected at the rate of 60 mg./kg. once or twice daily. This was found to be substantially non-toxic.

Observations. While showing essentially similar action to that of heparin in regard to failure of debris absorption, as illustrated by Fig. 4, inhibition of sprouting was less complete, as may be seen from Table 4A. The reduction is still, however, obvious, the mean of 1·8 p.c. contrasting with the comparable control figure of 8·5 p.c. after four days; similarly the seven-day figure of 7·8 p.c. contrasts with the control mean of 22·4 p.c. In the nerve trunks, Schwann cell hypertrophy was decreased, macrophages absent, and debris persisted.

Since these drugs might be regarded still as unconventional anti-mitotics, it was considered desirable to test the action of a substance better known for its anti-mitotic effects, but whose properties appear comparable with those of the muco-polysaccharides. Such a drug is colchicine.

(ii) *Colchicine.* This drug was injected in approximately 8 p.c. alcohol, as recommended by Fleischmann (1939), at first at the rate of 2–3 mg./kg. daily, but after toxic symptoms appeared the dose was reduced to 1 mg./kg. daily. No mortality resulted from this dosage. Nine animals were used in all, being killed seven days after operation. Results are tabulated in Table 5.

TABLE 5.

Re-innervation percentages in colchicine treated series. (7 days post-operative)

Animal	Re-innervation p.c.	Residual
Co. 1	6·6	49·3
Co. 2	2·1	63·0
Co. 3	10·6	54·0
Co. 4	9·6	42·9
Co. 5	(37·9)*	21·3
Co. 6	17·6	48·7
Co. 7	11·6	52·6
Co. 8	4·2	46·8
Co. 9	(36·6)*	46·3
Mean and confidence limits	13·8 / *S·3* / 4·2 (control mean 22·4)	
Odds favouring significant reduction:	25,000 : 1	

* Co. 5 and 9 showed a strongly atypical reaction (see text) and have not been included in the computation of the mean.

Observations. In all but two cases the action of this drug was similar to that of sodium alginate, both in character and degree. Sprouting was slowed down, and the mean of 8·3 p.c. re-innervation after seven days may be contrasted with the comparable control figure of 22·4 p.c., and comparing closely

with the seven-day figure for sodium alginate. Two animals, however (Co 5 and 9), showed far higher sprouting indices than any of the others, tending to rise above even those of the controls. They yielded re-innervation percentages of 37·9 and 36·6 respectively. The histological appearance of these two cases differed qualitatively from anything encountered previously: instead of, as is usually the case, only one sprout emerging from a given point on a residual fibre, five or more emerged together, as may be seen in Fig. 16, completely distorting the residual fibre at that point, and making difficult the detection of its distal portion from the surrounding sprouts. Under these conditions, sprouts often outnumber residual fibres in some locations, as is illustrated in Figs. 12, 14 and 15, while extensive distortion and varicosity is evident. Such instances complicate the determination of the rôle of the node in sprouting, since it is possible that the node is distorted out of recognition during formation of the sprout, and reconstituted later. This would, of course, be extremely difficult to establish.

Examination of this material suggests that sprouting has not occurred at more points on residual fibres than in control material, but that each sprouting locus forms more new collateral branches. The phenomenon is, however, qualitatively new, and provides interesting information concerning the effects of excessive sprouting on the residual nerve-fibre.

Examination of the nerve trunks revealed changes similar to those found with muco-polysaccharides—there were not the large numbers of conspicuous metaphase (equatorial plate) mitotic figures seen eight hours after administration of a single dose of colchicine (Fleischmann, 1939). Some metaphase figures were seen, but a number of Schwann nuclei were clumped and distorted, as described in colchicine-treated epidermis by Isler (1950), and in liver by Kantner (1951). Absorption of debris was again delayed.

(b) *Nuclear anti-mitotics.*

The indications from the foregoing experiments are that anti-mitotic substances of the "cytoplasmic" type may arrest both mitosis of Schwann cells and sprouting of intact axons. This result tends to vindicate the suggestions of Rexed and Rexed concerning re-innervation of the cornea, but several possibilities arise when one attempts to interpret these effects. These substances may act indirectly in delaying sprouting, either by preventing Schwann cells from reaching the hypertrophic state in which they might secrete the stimulator lipid, or by slowing down the release of this material from the degenerating nerve by interfering in the attendant cellular processes concerned, such as phagocytosis. Alternatively, the phenomena of mitotic inhibition and interference with sprouting may represent independent effects—the inhibitor may interfere directly in the response of the axon to neurocletic stimulation.

In an attempt to differentiate between these alternatives, recourse was had to another group of mitotic inhibitors, which act by a different mechanism.

These, the "pycnotic" or "nuclear" group, are represented here by bis-nitrogen mustard (methyl-bis-β-chlorethylamine hydrochloride). An alkaloid reported to possess anti-mitotic properties (Barnard, 1949; Cleland, 1950) has also been tested. This substance, cryptopleurine (de la Lande, 1948), has been included under the heading of "Nuclear" anti-mitotics, although its position is not clearly defined.

TABLE 6.

Re-innervation percentages in series treated with Pycnotic anti-mitotics (7 days post-operative)

(A) Bis-nitrogen mustard		
Animal	Re-innervation p.c.	Residual
NM 3	22·5	52·8
NM 4	8·6	49·8
NM 5	31·0	54·9
NM 6	27·0	42·4
NM 7	21·4	45·0
NM 8	28·8	29·4
NM 9	21·8	35·8
NM 10	26·1	39·9
NM 12	26·0	49·0
NM 13	26·1	40·6
NM 14	20·0	46·3
NM 15	25·2	25·6
Mean and confidence limits	27·7 / *23·5* / 19·9 (control mean 22·4)	
	No significant difference from controls.	
(B) Cryptopleurine		
Crypt 1	42·2	42·3
Crypt 2	30·2	52·7
Crypt 4	45·6	36·0
Mean and confidence limits	59·9 / *39·3* / 20·2 (control mean 22·4)	
Odds favouring significant increase:	1,600: 1	

Nitrogen mustard. All the animals used in this series, totalling twelve, were killed seven days after operation and the counts are tabulated in Table 6a. The first three animals are survivors of a group of seven which received 1 mg./kg. daily until toxic symptoms appeared, when the dose was reduced to 0·5 mg./kg. daily. The remainder of the series received 0·5–1 mg./kg. daily; all survived, although toxic symptoms appeared.

As may be seen from Table 6A, no significant effect could be detected, and the histological appearance of this material was normal. The nerve trunk, however, showed profound effects in regard to Schwann cell activation, and debris elimination. Many Schwann cells showed pycnotic nuclei—shrunken, clumped and pleomorphic. Thus, marked inhibition of Schwann cell mitosis may apparently be achieved without affecting sprouting of nerve-fibres.

(c) *Cryptopleurine.* Only three animals were used in a preliminary experiment, its results being intended merely for comparison with those from nitrogen mustard treatment or control groups. The animals received 2-3 mg./kg. every second day for seven days.

Observations. As can be seen from Table 6B, the figures suggest significant increase in comparison with either control or nitrogen mustard figures. The histological appearance tended to reflect the increased sprouting, resembling the electrically stimulated material rather than that of the aberrant colchicine cases, however. The nerve trunks revealed the now familiar pattern of failure of Schwann cell hypertrophy and mitosis, accumulation of debris and clumped and pleomorphic Schwann nuclei.

TABLE 7.

Design of experiment on interaction between cryptopleurine and electrical stimulation.

Batch 1		Batch 2	
Animal No.	Characteristics	Animal No.	Characteristics
1	4 days control	1	7 days control
2	7 days electrical stimulation	2	4 days electrical stimulation
3	4 days 0·75 mg. cryptopleurine	3	7 days 0·75 mg. cryptopleurine
4	7 days 0·75 mg. cryptopleurine + electrical stimulation.	4	4 days 0·75 mg. cryptopleurine + electrical stimulation.
5	7 days 1·5 mg. cryptopleurine	5	4 days 1·5 mg. cryptopleurine
6	4 days 1·5 mg. cryptopleurine + electrical stimulation.	6	7 days 1·5 mg. cryptopleurine + electrical stimulation.
7	7 days 3 mg. cryptopleurine	7	4 days 3 mg. cryptopleurine
8	4 days 3 mg. cryptopleurine + electrical stimulation.	8	7 days 3 mg. cryptopleurine + electrical stimulation.

Failure of an anti-mitotic dose of cryptopleurine to inhibit sprouting is, of course, significant, but it was felt that the increased sprouting merited further investigation. Accordingly a further experiment, designed by Dr. F. E. Binet and set out in Table 7, was carried out. Sixteen animals in two batches were used; they originated from the same hybrid population as those used in the dye-feeding experiments, and within each batch the animals were as closely related as possible. In this experiment, electrical stimulation (fifteen minutes' duration, with a current of 1·5 mA, fifty cycles frequency), was combined with four levels of drug dosage: 0,0·75 mg./kg., 1·5 mg./kg., 3·0 mg./kg. every second day. Unfortunately the results were rendered incomplete in group 2 by death of No. 8, failure of material to stain satisfactorily in No. 3 and accidental administration of one dose of cryptopleurine to No. 2.

Despite these complications, the results have been analysed and have provided some interesting features. Most likely re-innervation percentages, together with 95 p.c. confidence limits, have been computed, and are set out in Table 8B. From these it will be seen that cryptopleurine in smaller doses produces a marked and consistent acceleration of sprouting, but that the maxi-

mal dose (3 mg./kg.) produces a decreased effect. Electrical stimulation produced a possible further acceleration when combined with cryptopleurine treatment—odds of 15 : 1 favoured this, but are insufficient to establish it.

TABLE 8.

(A) *Results of the experiment set out in Table 7.*

Treatment	Animal	4 day Re-innervation p.c.	Animal	7 day Re-innervation p.c.
Con.	B1(1)	7·8	B2(1)	17·5
E	B2(2)	5·5*	B1(2)	25·0
Crypto. 0·75	B1(3)	21·2	B2(3)	—†
Crypto. 1·5	B2(5)	9·0	B1(5)	53·5
Crypto. 3	B2(7)	1·5	B1(7)	43·6
E+Crypto. 0·75	B2(4)	15·5	B1(4)	32·0
E+Crypto. 1·5	B1(6)	7·0	B2(6)	70·0
E+Crypto. 3	B1(8)	6·3	B2(8)	—‡

(B) *Most likely values for percentage of re-innervation following administration of varying doses of cryptopleurine.*

Cryptopleurine mg./kg.	4 day	7 day
	11	15
0	*5·7*	*9·0*
	2	4·5
	23	48
0·75	*15·4*	*38·8*
	9	29
	20	65
1·5	*13·1*	*55·9*
	7·5	46
	5·5	63
3·0	*2·1*	*53·4*
	0·5	43·5

* Inadvertently received a dose of cryptopleurine.
† Material failed to stain satisfactorily.
‡ Died during period of treatment.

TABLE 9.

General summary of experimental results.
Mean percentage re-innervation after various treatments.

Treatment	4 day	7 day	14 day
Pyronin	41·7 (control 5·8)	80·8 (control 24·7)	
Cryptopleurine (first series)		39·3	
Electrical stimulation	23·5	32·2	
Bis nitrogen mustard		23·5	
Control	*8·5*	*22·4*	*44·5*
Colchicine		8·3	
Sodium alginate	1·8	7·8	29·1
Heparin	0·5	2·7	

The dual effect—acceleration in small doses and inhibition in larger ones—is quite striking, and contrasts sharply with the complete failure of nitrogen mustard, in toxic dosage, to influence sprouting. The occurrence of significant acceleration when doses of anti-mitotic level are administered, however, supports the alignment of these data alongside those for nitrogen mustard.

DISCUSSION.

The results from electrical stimulation experiments lead to the conclusion that the entire cellular synthesizing system (nucleolus, nucleolar satellite and cytoplasmic ribonucleic acid-containing bodies) may be readily activated, accelerating axoplasmic synthesis which in turn affects the rate of formation of new sprouts. This latter effect is presumably achieved, either by increasing availability of axoplasm, which has been drained off into new sprouts, or by increasing the turgor of the cell, which von Muralt (1945, 1950) suggests regulates the behaviour of peripheral portions of the neurones. If, in fact, these changes occurring in the metabolic activities of the perikaryon result in increased axoplasmic synthesis and pressure, axoplasm will be more readily available for the extrusion of new sprouts, and depletion of the parent fibre, by excessive sprouting, is less likely to occur.

No direct correlation has been established between proportion of denervation and rate of re-innervation, but Edds and Small (1951) found that in monkeys severe denervation was followed by little sprouting, although sprouting was substantial when moderate denervation was achieved. Further, as has been pointed out previously (Hoffman, 1951) maturation of sprouts, which must involve the supply of axoplasm from the perikaryon, is delayed in regions of excessive denervation, where each residual fibre forms many sprouts. This evidence tends to support the view that there are limitations to the rate of supply of axoplasm to the periphery.

Several workers have recently suggested that axoplasm actually flows perpetually to the periphery. Young (1942, 1945) has based his conclusions on the differing behaviour of the central and peripheral cut ends of interrupted nerves: Weiss and Hiscoe (1948) severed nerves and placed constricting cuffs (made from arterial segments) around them. At first the axoplasm was expelled equally into central and peripheral portions of the axon, but gradually the proximal side swelled while the distal side thinned out. This led them to suggest that proximo-distal flow had occurred; the interpretation has been further considered by Weiss (1950a). Gerard (1950) has shown that phosphoprotein, incorporating injected radio-active P^{32}, advances down the nerve at approximately the rate defined by Gutmann *et al.* (1942) for regeneration of the axon. If, then, a continuous flow occurs, when new sprouts are formed the neurone must increase the supply of axoplasm—not only for the formation of new sprouts, but also for their maintenance. It therefore seems reasonable to suggest that the effect of electrical stimulation is, primarily, to accelerate the

synthesis of axoplasm by the perikaryon, thus enabling a more rapid response to peripheral "neurocletic" stimulation of the axon.

The results obtained in these experiments following electrical stimulation tend to conflict with the conclusions of Liu-Chan, Bailey and Windle (1950), who suggested that the only changes occurring in neurones following electrical stimulation may be explained in terms of axon trauma. It is obvious that the acceleration observed here must follow changes brought about in intact neurones.

Whilst even less is known about the effects of Pyronin *in vivo*, certain observations tend to suggest that the accelerator effect which this substance has exerted on axon sprouting may also be explained in terms of modification of synthetic processes in the perikaryon. Brachet (1940) has shown that the structures stained by Pyronin in sections of fixed tissues are those containing ribonucleotides. Although the experiments of Kurnick (1950) and Kurnick and Mirsky (1950) have established that the specificity of Pyronin is for depolymerized nucleic acid as against polymerized, rather than for the sugar contained (desoxyribose or ribose), *in vitro* experiments of Clark and Gunson have shown that only ribonucleic acid containing structures in the living cell —basal granules in the rat pancreas, nucleolus is Drosophila salivary gland— stain with Pyronin, while depolymerization with acid renders chromosomes stainable. Thus it may be suggested that Pyronin attaches specifically to the ribonucleic acid bodies, such as Nissl granules in the neurone, and presumably modifies the mechanism by which protein is synthesized.

In regard to the rôle of Schwann cells in axon sprouting, the results obtained with mitotic inhibitors clearly indicate that there is no direct correlation between mitosis of these cells and the initiation of sprouting. All of the anti-mitotic substances used inhibited the hypertrophic and hyperplastic reactions of Schwann cells, which normally accompany Wallerian degeneration; in all the animals treated with inhibitors, disintegration and absorption of the degenerated nerve-fibres was retarded. This may be due partly to the inactivation of invading or resident macrophages, but it is also possible that these drugs may inhibit the transformation of Schwann cells into macrophages described by Weiss (1944) and Weiss and Wang (1945); the decreased numbers of macrophages observed under these conditions supports this suggestion. Differences in the appearance of Schwann cells treated with different inhibitors may, of course, be detected; colchicine and the muco-polysaccharides produce less distinctive nuclear changes than do the pycnotics, while cryptopleurine produces pycnotic changes of less marked character than does nitrogen mustard.

When one turns to the effects exerted on axon sprouting, a sharp dichotomy in action becomes apparent. The "cytoplasmic" group, in general, exerted a marked inhibition of sprouting—although two of the colchicine-treated animals behaved quite atypically. The "pycnotic" or "nuclear" (nitrogen mustard) failed to inhibit sprouting when administered in anti-mitotic doses, while cryptopleurine produced marked acceleration, except in very large doses.

In view of these marked differences, the inhibition of sprouting must be attributed to a direct interferenec with the sprouting mechanism, while the failure of the "nuclear" inhibitor to affect the process must be correlated with the differing mode of interference with the mitotic cycle. Nitrogen mustards are known to interfere with nuclear processes—to damage chromosomes (Bodenstein, 1947) and to interfere in the synthesis of desoxyribonucleic acid (Herriot, 1951). The action of cryptopleurine has not been defined with equal clarity. Barnard (1949) described essentially colchicine-like action on onion root tips, concluding that "For cytological purposes cryptopleurine would seem to be an excellent substitute for colchicine" while Cleland (1950) observed marked contraction of the chromosomes and pycnotic changes in Ostrea eggs, concluding that no colchicine-like activity was evident. Barber (unpublished) in an extensive analysis, concludes that, although differences from nitrogen mustard are detectable, essentially cryptopleurine is a pycnotic, and in fact slowly kills the dividing cells. The results obtained in these experiments confirm that cryptopleurine, applied repetitively over a long period, produces pycnotic changes in cell nuclei: however, the duality of effect on sprouting rate, produced according to dose level, suggests significant differences from a typical pycnotic drug, such as nitrogen mustard. Superficially this dual effect might be compared with that of colchicine where, it will be recalled, of nine animals treated, seven showed significant inhibition, while two produced increased sprouting. This comparison is not supported by histological pattern, however, and the mechanism of acceleration is therefore presumably different. The inhibition by cryptopleurine might conceivably be due to a toxic effect (compare Barber's "slow death of cells") but the negative results obtained with toxic doses of nitrogen mustard tend to negate this suggestion.

In regard to the action of pycnotic drugs, some observations of Edds (personal communication) are of interest. He has found that X-irradiation of the partially denervated limb, while substantially inhibiting Schwann cell mitosis and debris elimination, has no effect on sprouting. In view of the conclusions of Boyland (1948) and Boyland *et al.* (1948) that nitrogen mustards closely duplicate the biological effects of X-irradiation, these results support the failure to correlate Schwann cell mitosis and axon sprouting.

Inhibition of sprouting by the "cytoplasmic" anti-mitotics requires more extensive consideration. The mode of action of colchicine and related drugs has been studied intensively: Lüscher (1945) applied colchicine to Tubifex eggs and found that, if applied in interphase, spindle formation is inhibited and mitosis arrested in metaphase. If applied after spindle formation has commenced, mitosis is completed normally and arrested in the following metaphase. Beams and Evans (1940) reported that in Arbacia eggs colchicine resulted in lowering, or prevention of increase, of cytoplasmic viscosity following fertilization: cleavage was thus inhibited. In a study of viscosity changes in Chaetopterus eggs during cleavage, Heilbrunn and Wilson (1948) found that

the appearance of the spindle was preceded by a marked increase in cytoplasmic viscosity, which they considered was a form of gelation. In a further study of the rôle of heparin in suppressing cleavage, the latter authors (1949) found that it suppressed this characteristic gelation. Harding (1949) showed that heparin had similar effects on artificially-activated mitosis of frog eggs, and Most (1951) has shown that the anti-mitotic bacterial polysaccharide (Shear's polysaccharide) has heparin-like anti-coagulant properties. Harding (1951a) has demonstrated that a range of muco-polysaccharides behave as "cytoplasmic" anti-mitotics.

It therefore appears that colchicine and the muco-polysaccharides inhibit cleavage by interfering in the gelation of plasmasol associated with spindle formation. Interference with the mechanism of sprouting may be interpreted in terms of similar processes. As has been pointed out earlier (Hoffman and Springell, 1951) sprouting presumably follows the local penetration of the axolemma by a biologically active lipid, resulting in local liquefaction of the axogel and streaming of the axosol through the resulting aperture, gelling on the sides as it escapes. Interference with the local responses of the protoplasmic colloids, by administering an anti-gelling agent (such as one of the "cytoplasmic" anti-mitotics) might be expected to inhibit this "neurocletic" response. The results of Lorch and Goldacre (1950) support this hypothesis: they injected heparin into an amoeba and observed prompt liquefaction at the point of injection, extrusion through the aperture of the contents of the amoeba, followed by failure of the cortical plasmasol to gelate, and cessation of locomotion. From these observations it may be concluded that the anti-gelling agent liquifies the axogel, prevents its restoration and therefore inhibits outgrowth of sprouts.

If this hypothesis is correct, one may now consider the possible causes of the two atypical responses to colchicine. If, in these cases, the lowering of viscosity of the cortical gel did not reach a critical level required to inhibit locomotion, the gel would merely be rendered more sensitive to local breakdown: in effect the colchicine would synergize the locally-acting "neurocletic" lipid. Outflow of axosol would still occur, but lateral gelation would ensue less readily, and axosol might escape in many directions, thus forming the multiple sprouts described above.

In previous discussion (Hoffman and Springell, 1951; Edds, 1950) the problem of localization of the neurocletic response has been raised; the results obtained here enable certain conclusions to be drawn. The regular occurrence of sprouting at nodes prompted two alternative suggestions: either Speidel's phenomenon of node formation, by Schwann cell mitosis and separation of daughter cells, regularly precedes sprouting, or else the occurrence of sprouting is substantially restricted to the nodal region. Since inhibition of Schwann cell mitosis does not necessarily retard sprouting, the latter conclusion seems

inevitable. The special sensitivity of the node to neurocletic stimulation may result from the axon being bare in this region—unshielded by Schwann protoplasm—thus permitting more ready penetration by the active lipid. The localization of responsiveness to a small fraction of the axonal surface would partly explain the absence of surplus sprouts and the extremely ordered pattern of sprouting, since if only one sprout formed at each node, the maximum number which could be formed would be strictly limited.

SUMMARY.

Electrical stimulation of the spinal cord or nerve trunks significantly accelerates the re-innervation of denervated fibres, by the sprouting of intact axons, in partially denervated muscles. The time relations of the onset of acceleration closely parallel those reported for the changes in Nissl substance of the nerve cell bodies. Feeding of the dye Pyronin produces an even more marked acceleration of re-innervation, although other dyes of similar molecular configurations fail to do so.

Anti-mitotic drugs, which inhibit the characteristic mitotic and hypertrophic changes occurring in Schwann cells following Wallerian degeneration, fall into two groups, according to their effect on rate of sprouting of axons. The "cytoplasmic" group (colchicine and muco-polysaccharides) retard sprouting, whilst the "nuclear" or "pycnotic" group (represented by bis-nitrogen mustard) is without effect. Administration of the drug cryptopleurine, known to be anti-mitotic but of doubtful classification, produces acceleration or retardation, according to dose.

A general hypothesis has been advanced to explain these observations; electrical stimulation is believed to increase sprouting rate by accelerating axoplasmic synthesis in the cell body. Pyronin is tentatively considered to act similarly. The "cytoplasmic" anti-mitotics are believed to inhibit sprouting by reducing the viscosity of the cortical layers of the axon (axogel), thus preventing pseudopod extrusion.

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SEROLOGICAL STUDIES OF THE MULTIPLICATION OF A STAPHYLOCOCCAL BACTERIOPHAGE

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In a previous paper (Rountree, 1951a) it was reported that complement fixation occurred when coli bacteriophage T5 reacted with its homologous antiserum in the presence of complement. By cyanide lysis during the latent period, T5 infected cells were shown to synthesize antigenic material reacting in the complement fixation test at a stage in the phage growth cycle at which few or no infective phage particles could be demonstrated. It was postulated that the final step in phage multiplication, i.e. the production of complete infective particles takes place in the last part of the latent period after a pool of this antigenically reacting material has been accumulated.

In the present paper an account is given of the production of antiphage sera in rabbits and of the use of these sera in studying the phenomena of phage adsorption and multiplication. A staphylococcal phage, 3A of Wilson and Atkinson's (1945) series of typing phages, and its host strain, 284, have been used since it was desired to see if results comparable with those obtained with the T5—coli system could be obtained with a different phage—host system. No evidence of lysogenicity in strain 284 was obtained in a previous study (Rountree, 1949a). The presence of lysogenicity cannot, however, be excluded since a suitable indicator strain for a carried phage may not be available.

METHODS.

Phage counts were made by the surface agar plating method, each plate accommodating 4 inocula of 0·01 ml. each.

Phage adsorption was measured by titration of free phage in the supernatants of infected broth cultures centrifuged at various times after addition of the phage.

Phage neutralizing antibody was titrated by the method of Burnet, Keogh and Lush (1937).

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The technique of the complement fixation test was that previously described for the coli-T5 system (Rountree, 1951a).

Broth cultures were grown in 0·15 p.c. glucose veal broth in a water bath at 37° C. and aerated during growth by bubbling oxygen through them.

All phage and cell dilutions used in making viable counts were made in broth held in an ice water bath.

Numbers of bacterial cells in broth cultures were determined by a turbidometric method using a "Unicam" photoelectric colorimeter equipped with an Ilford 404 green filter. Turbidometric standards were established by checking colorimeter readings against viable cell counts made by the method of Miles and Misra (1938). One difficulty inherent in working with staphylococci is the morphological arrangement of the organisms. In young broth cultures the proportion of many-celled clumps will be small but at all phases of growth the viable cell count will be lower than the actual number of viable coccal units present. Samples for microscopic examination were therefore taken at the same time as the samples for turbidity reading and viable count. After staining, the proportions of the numbers of cocci occurring singly and in pairs, fours, sixes and eights were determined in each sample and the necessary corrections applied to the viable counts. With cell concentrations ranging from 4×10^7/ml. to 4×10^8/ml. there was good agreement between the corrected cell counts and the turbidity expressed as log I/I_0, the plots of these values falling on a straight line.

Characteristics of Phage 3A.

Morphology. Electron microscopy (Farrant and Rountree, 1952) shows phage 3A to consist of an oblong flat head approximately 560Å in width and 950Å in length and a long tail 2,700 to 3,000Å in length and 80–100Å in width.

Adsorption. Divalent cations are required for adsorption of phage 3A to its propagating strain (Rountree, 1951b). The optimal concentration of $CaCl_2$ or $MgCl_2$ permitting 90 p.c. adsorption in 20 minutes at 37° C. is 10 μg/ml.

In glucose veal broth cultures the rate of adsorption, measured by the disappearance of free phage from the supernatant, is that of a first order molecular reaction (Puck, Garen and Cline, 1951). Values for the adsorption constant K, using various times and phage and cell concentration, are given in Table 1. Within 5 minutes of adding phage to a bulk broth culture at a ratio of 3–4 particles per cell, 98 p.c. of the phage will have been adsorbed and multiple infection established.

The one step growth curve.

Various methods are available for determining the one step growth curve of a phage. In one method, sufficient phage is added to infect every cell in the broth culture and at intervals samples are removed for suitable dilution and plating for the phage count. This technique involves the use of numerous dilution tubes and, while it must be used for determining the burst size and in cyanide lysis studies, a simpler method can be used to determine the minimum latent period and the end of the one step growth curve. In the simpler method, sufficient phage is added to the broth culture to give only 2–4 plaques when

0·01 ml. samples taken during the latent period are plated directly. In such a culture there will be a great excess of cells over phage and phage released at the end of the latent period will be almost immediately readsorbed on to uninfected cells. Two or more particles may adsorb on to one cell and, furthermore, owing to the morphological arrangement of the cocci, a pair of cocci in which both cells are infected will yield only one plaque when plated. The simpler method is therefore unsuitable for estimating the average yield of phage per cell as the value obtained is always lower than that obtained when every cell in the culture is infected.

TABLE 1.

Adsorption of phage 3A in glucose veal broth.

Cells/ml. × 10^8	Phage/ml. × 10^8	Time in minutes	p.c. Adsorption	K.cm^3/min.$^{-1}$
2·35	9·4	5	98	$3·19 \times 10^{-9}$
2·6	8	5	97·7	$2·92 \times 10^{-9}$
1·8	5·8	10	94·1	$1·56 \times 10^{-9}$
2·5	16	13	99·6	$1·70 \times 10^{-9}$
3·3	6·8	15	99·4	$1·02 \times 10^{-9}$
2·6	8	15	99·2	$1·22 \times 10^{-9}$

$K. = \frac{2·3}{(B)\,t} \log \frac{Po}{P_t}$ where (B) = bacterial numbers/ml.,

t = time in minutes, P_0 = phage free at time o and P_t = phage free at time t.

Using the simpler method, it was found that results with non-aerated broth cultures were erratic in that in many experiments the phage failed to multiply. Further observations (Barbour, 1951) showed that many of the cells in these cultures were resistant to phage infection in the sense that adsorption of the phage was followed neither by growth of phage nor by death of the cells. However, when the cultures had been grown with aeration, one step growth curves were regularly obtained with a latent period of 50 minutes, the step being completed at 75 minutes.

There was a specific Ca^{++} requirement for phage growth. In the absence of added Ca^{++}, in broth that had been adequate for adsorption, there was a loss of infective phage, a variable increase in the length of the latent period usually amounting to 5–10 minutes and a reduced average burst size. The addition of 200 μg/ml. of $CaCl_2$ just prior to phage infection resulted in maximal phage production.

Most of the work to be described in the later part of this paper was concerned with one step growth curves in broth cultures of 100 ml. or more. Some

details must therefore be given of the results obtained when lysis of bulk cultures was followed turbidometrically and by plaque count.

In these cultures, the cells were grown with aeration to a concentration of 1–2 $\times$ 10^8 cells/ml., $CaCl_2$ and sufficient phage to infect every cell were then added separately and samples for phage count and turbidity readings taken at suitable intervals.

The essential features of the growth curve thus obtained are shown in Fig. 1 (curve A). In this particular instance, the initial ratio of phage to cells was 3 : 1. The first release of phage occurred at 50 minutes but the major portion (90 p.c.) of the phage yield was released between 80 and 90 minutes. The average burst size was 120 particles per cell.

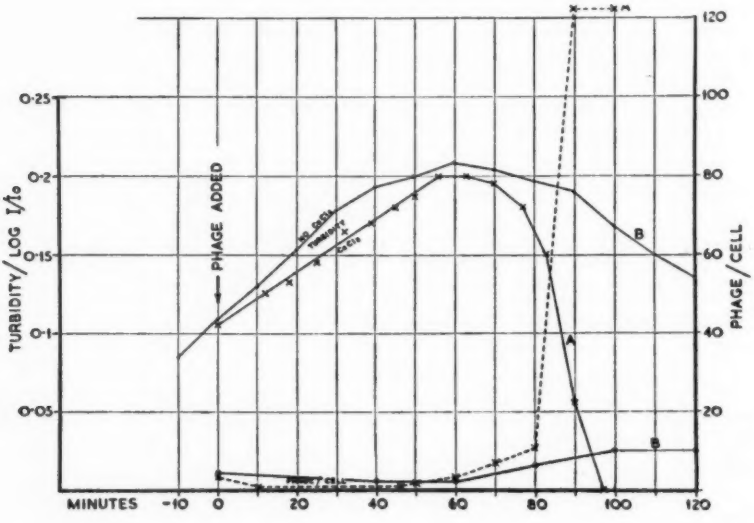

Fig. 1. One step growth curves of phage 3A in aerated glucose veal broth. A, with added $CaCl_2$, 200 μg/ml.; phage/cell ratio, 3 : 1. B, no added $CaCl_2$; phage/cell ratio, 4:1.

The curve of the turbidometric changes is of considerable interest. For 55 minutes after the addition of the phage there was a constant increase in the turbidity of the infected culture. There followed a short period during which the turbidity remained constant. After 63 minutes it commenced to fall, slowly at first and then at an increasing rate between 80 and 90 minutes. The culture was clear at 95 minutes.

The increase in turbidity following infection corresponds to what would represent a doubling of cell numbers in an uninfected culture and has been

constantly observed in all one step growth curves with a multiplicity of infection from 2–7 particles per cell. This increase in turbidity differs strikingly from the findings with coli-T phage systems and with the *Staph. muscae* phage system studied by Price (1948) in which addition of phage immediately halted the turbidity increase. It was therefore important to ascertain whether this apparent increase in cell numbers following infection with phage 3A was due to the actual multiplication of the cells or was merely an optical effect. Measurement of the diameter of infected cocci taken at various times during the latent period and stained with Gram revealed no significant increase in cell diameter. Formalin was added to other cell samples to prevent further growth of cells or phage and direct microscopic counts were made. These showed that there was no actual increase in cell numbers during the latent period. The increase in turbidity must therefore be due to changes in the optical properties of the cells in which phage growth is taking place.

TABLE 2.

Relationship of the time of clearing in mass cultures to average burst size.

Added $CaCl_2$ μg/ml.	Initial cells × 10^8/ml.	Initial phage × 10^8/ml.	Ratio phage /cells	Commencement of clearing. minutes	End of clearing. minutes	Average burst size
200	1	7	7	60	88	120
200	1·8	10	6	62	90	96
200	1·35	5·3	4	68	98	120
200	1·8	5·8	3	68	97	120
200	1	5·5	5·5	68	98	95
200	1·1	4·5	4	73	100	93
200	1·5	4·4	3	83	140*	53
nil	1·9	5·3	2·7	75	205*	10
nil	2·0	7·4	3·7	75	200*	9

* clearing not complete.

Table 2 gives the results of a number of growth curves. In some experiments there were variations in the time of commencement of clearing and also in the times at which clearing was complete. In certain experiments the cultures never cleared completely. The prolongation of the clearing time was correlated with a low burst size and was especially marked when no $CaCl_2$ was added to the medium. In the absence of $CaCl_2$ there was an average burst size of only 10 particles per cell. This phage was all released by 100 minutes. Fig. 1 (curve B) shows the slow fall in turbidity until 140 minutes, at which point clearing stopped. Smears taken from such cultures at 100 minutes did not, however, reveal any intact cocci and only Gram negative debris was seen.

These results suggest that the clearing of broth cultures with this particular phage may be due to two distinct but related processes. The first process is the release of phage from the infected cells. On its termination the cells

had lost their ability to retain the Gram stain but the turbidity readings indicated that they had not completely disintegrated. The second process is the solution in the medium of the disintegrating remnants of the infected cells. In cells that have given a low yield of phage this terminal process of solution is incomplete and complete clearing of the culture does not occur. One possible explanation of this may be that the remnants of cells with low yields are not sufficiently small to be easily soluble. Another possibility is that disintegration of the cells is due to an enzymic action by the phage particles and that in cultures with poor phage yields the final concentration of particles will not be sufficiently high to accomplish this disintegration of the cells. Insufficient data on the mechanism of lysis are available at present to decide whether either of these explanations is correct.

Production of Antiphage Sera.

The production of complement fixing and phage neutralizing antibody was studied in a series of rabbits immunized with phage 3A. A single stock of the phage prepared in broth was used for the injections.

Like the other typing phages, this phage has customarily been prepared by extraction of cultures lysed on agar plates and subsequent filtration of the extracted fluid through a gradocol membrane. Such filtrates are unsuitable for use in complement fixation reactions since they contain particles of agar which render them anti-complementary. Cultures lysed in broth must therefore be used as antigen in the complement fixation tests.

Immunization of rabbits.

In a preliminary experiment, two rabbits, R201 and R202, received intravenously the doses of phage shown in Table 3. Fourteen days after the first injection, trial bleedings were made and the complement fixing and phage neutralizing antibody titres determined. Complement fixing antibody at a titre of 1 in 190 was found in both sera and R201 was bled on the following day to provide a stock of serum for complement fixation tests with various phage antigens and for "chessboard" titrations with 3A.

R202 then received two further injections of antigen at intervals of one week and further samples of sera were obtained from both rabbits on the 28th and 61st days of the immunization.

The antibody titres in Table 3 indicated definite differences in the behaviour of the antibody when measured by the two methods. In both animals the complement fixing antibody had reached its highest titre (or perhaps was falling) by the 14th–15th day. In R201 the titre fell to 1 in 24 by the 28th day. In R202 which received further doses of antigen it was still at the 1 in 196 level on the 28th day but in this animal also it subsequently fell. On the other hand,

the neutralizing antibody was rising at a time when the complement fixing antibody remained stationary or was falling.

These results lent strong probability to the view that two phage antibodies (and by inference, two antigens) were involved and the antibody response in further rabbits was accordingly investigated.

TABLE 3.

Neutralizing and complement fixing antibody titres in rabbits immunized with phage 3A.

Day	Rabbit 201			Rabbit 202		
		Antibody titre			Antibody titre	
	Dose of antigen	Neut.*	C.F.†	Dose of antigen	Neut.	C.F.
0	$2{\cdot}5 \times 10^9$			$2{\cdot}5 \times 10^9$		
4	5×10^9			5×10^9		
7	$7{\cdot}5 \times 10^9$			$7{\cdot}5 \times 10^9$		
14					100	190
15		200	190	$1{\cdot}3 \times 10^{10}$		
21				2×10^{10}	2,000	196
28		20,000	24		20,000	196
61		7,000	24		16,000	32

* Reciprocal of dilution giving 80 p.c. neutralization of phage.

† Reciprocal of dilution giving 50 p.c. haemolysis with excess antigen and 2 MHD complement.

Response to a single dose of antigen.

The primary response to a single dose of antigen was followed in 3 rabbits, R204, R205 and R206, each being given $1{\cdot}3 \times 10^{10}$ particles intravenously. The responses of R204 are shown in Table 4, and of R205 and R206 in Fig. 2. They indicate that one dose of the phage was an efficient antigenic stimulus.

TABLE 4.

Primary and secondary response of R204 to phage 3A following single doses of phage.

	Primary response				Secondary response		
	Antibody titre				Antibody titre		
			C.F.				C.F.
Day	Neut.	Unadsorbed	Adsorbed	Day	Neut.	Unadsorbed	Adsorbed
0	Nil	14	3	1	5,500	24	24
2	Nil	14		2	6,000	24	
4	2	24		3	12,000	48	
6	70	48		4	50,000	64	
9	400	128		7	400,000	96	
13	900	128		9	400,000	96	96
64	5,500	24		11	200,000	96	
				14	200,000	96	
				32	160,000	n.d.	
				49	80,000	64	

Complement fixation was obtained with sera removed before administration of the antigen. Adsorption of these sera with large doses of 284 cells removed or reduced this antibody, showing it to be antistaphylococcal in nature. Complement fixing antibody obtained later in immunization was not reduced by adsorption.

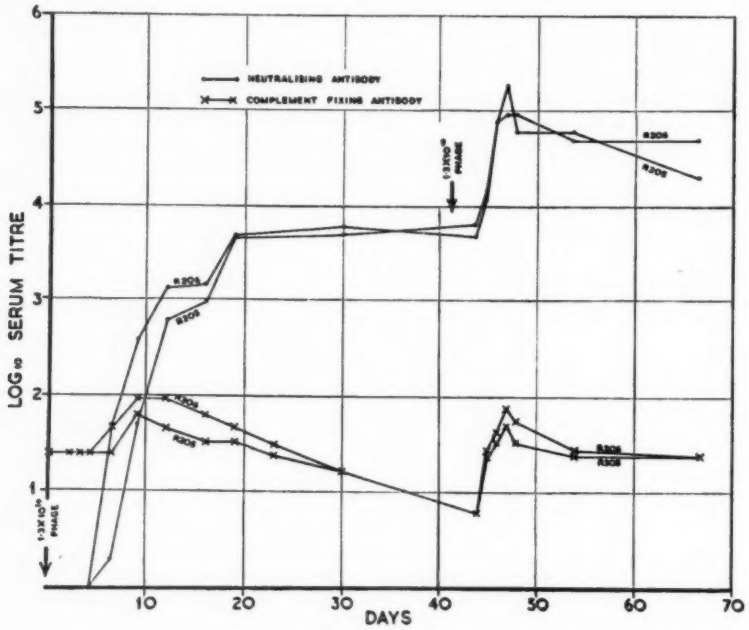

Fig. 2. Production of complement fixing and phage neutralizing antibody in rabbits R205 and R206, immunized with phage 3A.

Furthermore, the rise in complement fixing antibody commenced on the 4th or 5th day and not earlier as would have occurred had the initial complement fixing titre been due to previous antigenic experience of the phage or had the rise in complement fixing titre been due solely to the production of staphylococcal antibody.

The rise in complement fixing antibody continued until the 9th day and thereafter remained stationary in R204 and R206 till the 12th or 13th day, when it commenced to fall. In R205 it fell from the 9th day onwards.

The rise in neutralizing antibody likewise commenced on the 4th or 5th day. However, it continued until the 19th day and remained at this level until the animals were tested for their secondary response.

The secondary response was elicited in these 3 animals and in R201 and R202 by a further dose of $1{\cdot}3 \times 10^{10}$ phage particles. For both antibodies, it

was of the accelerated logarithmic type described by Burnet and Fenner (1949) for the coli dysentery phage, C 16. The titres obtained are shown in Tables 4 and 5 and in Fig. 2.

Although the 5 rabbits showed individual differences in the magnitude of their antibody responses, the general outline of antibody production was essentially the same in all the animals. The point, however, that is of particular interest is that there was no correlation between the complement fixing and neutralizing antibody values. This is illustrated clearly in Fig. 2.

Even in rabbits R201 and R202 with the extraordinarily high neutralizing antibody titres of 1 in 2,000,000 the complement fixing antibody titres did not rise further than 1 in 192, values already reached in the first course of immunization when the neutralizing antibody was only 1 in 20,000.

TABLE 5.

Secondary response in Rabbits R201 and R202 following one dose of 1×10^{10} *phage particles.*

Day after injection	Rabbit 201 Antibody titre		Rabbit 202 Antibody titre	
	Neut.	C.F.	Neut.	C.F.
0	3,000	24	16,000	14
1	3,000	24	16,000	14
2	3,000	24	16,000	12
3	14,000	80	70,000	48
4	100,000	160	500,000	192
7	1,600,000	192	1,300,000	192
9	1,600,000	160	2,000,000	192
11	1,600,000	128	1,600,000	192
14	2,000,000	96	1,600,000	192
32		96	170,000	48
49	500,000	64	130,000	24
63	500,000		130,000	24

The results obtained with these rabbits suggest that courses of immunization designed to give high titre neutralizing antisera will not necessarily give high titre complement fixing antisera, and that close attention should be paid to the time of bleeding if good complement fixing antisera are required.

Complement fixation with R201 serum.

"Chessboard" titrations (Hoyle, 1945) were carried out with the stock R201 serum using as antigen a broth lysate of 3A containing $2{\cdot}1 \times 10^{10}$ infective particles/ml. Fig. 3 gives the titration curves with 2, 4 and 6 MHD complement, using one hour's fixation at 37° C. and taking 50 p.c. haemolysis as the end point. The curves are similar in shape to those obtained with the T5 antigen antibody system and show no zones of optimal antigen or antibody concentrations. Since there was no flattening of the curve with the highest concentrations of antigen there was a possibility that the antibody end point had not been reached. It was not possible to prepare phage filtrates of infective titre higher than those used in these titrations.

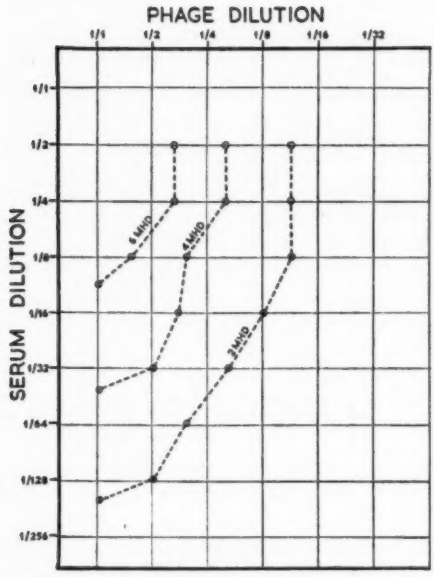

Fig. 3. Complement fixation with rabbit serum R201, phage 3A containing 2×10^{10} infective particles and 2, 4 and 6 MHD complement. Unadsorbed serum.

The serum was adsorbed with 284 cells, using 6 ml. of serum and the overnight growth from 10 agar plates washed off in saline and then concentrated by centrifugation. After filtration through a gradocol membrane this adsorbed serum was partially anticomplementary at a dilution of 1 in 4. Titration of the serum by the "chessboard" technique showed that the maximal antibody titre had been reduced to 1 in 128 but that this titre was obtained with antigen dilutions of 1 in 2 and 1 in 4 as well as with the undiluted phage. It appeared therefore that the serum had contained some staphylococcal antibody. Broth filtrates containing 5×10^9 infective phage particles/ml. should contain sufficient antigen to give maximal antibody titres with the adsorbed serum. Furthermore, the antiserum at a dilution of 1 in 8 should give as high an antigen titre as the undiluted antiserum. The adsorbed serum was used in subsequent titrations.

Specificity of the complement fixing antibody.

Previous studies (Rountree, 1949b) of the serology of the staphylococcal phages differentiated them into a number of serological groups between which there are few or no cross reactions. The basis of this differentiation is the infectivity neutralizing antibody and, since the results obtained with the present series of rabbit antisera had indicated the possibility of two antibodies being present in these sera, a number of other phages were used as antigens in complement fixation tests. Not all the phages were examined in this way, chiefly owing to the difficulty of preparing broth filtrates of sufficiently high titre to give the maximal antibody titre. The maximal antibody titre of adsorbed serum R201 using undiluted phages of titres of at least 5×10^9/ml. is given in Table 6. The neutralizing antibody titres obtained with these phages and a group A antiserum are included in the Table to show the serological specificity of this antibody. All phages of group A gave the same complement fixing antibody titre but in addition there were marked cross reactions with the phages of group B and with phage K.

TABLE 6.

Reactions of various Staphylococcal phages with a high titre Group A neutralizing antiserum and with the complement fixing antibody of R201 serum.

Phage	Serological group	Neutralizing antibody titre	Complement fixing antibody titre
3A	A	20,000	128
3B	A	20,000	128
47	A	20,000	128
51	A	20,000	128
29	B	<2	24
31B	B	<2	24
42D	B	<2	24
52	B	<2	24
K	D	0	32

These cross reactions provide further evidence for the view that the two methods used for titrating antibody are not simply different ways of measuring the same substance and serve to confirm the inferences drawn from the rabbit immunizations that the sera contain two distinct antibodies.

The Process of Adsorption Examined Serologically.

The first step in the process of phage multiplication is the adsorption of the infective particle to the cell surface. This having occurred, it has been presumed that the phage then penetrates the cell, crossing the barrier of the cell membrane by a process not understood but possibly enzymic in nature, and sets in train that series of syntheses which lead to the production of fresh phage.

There is the possibility, suggested by electron microscope photographs (Anderson, 1951) that the envelope and tail of the phage do not penetrate the cell but remain on the cell surface while the electron denser material of the head disappears, presumably into the cell, shortly after adsorption. It was suggested to me by Sir MacFarlane Burnet that serological evidence of the persistence on the infected cell's surface of the phage tail and/or envelope should be obtainable.

It has long been known (Burnet, 1933) that killed bacteria or bacteria in the resting stage to which 20–200 phage particles have been adsorbed can be agglutinated by antiphage sera. In such cells penetration of all the adsorbed particles probably does not occur. Nothing, however, is known of the agglutinability by antiphage sera of cells in the living actively metabolizing stage of growth at which they are fully susceptible to phage attack and infected with one or two phage particles only. Experiments were therefore designed to answer the question whether the entire phage particle penetrates such cells or not.

Experimental method.

Since adsorption without penetration would be expected to give agglutinable cells, it was important to ensure that infection was established in all the cells tested for agglutinability.

The method used for preparing the infected cells was as follows:

100 ml. of glucose veal broth were inoculated with 0·2 ml. of an overnight broth culture of strain 284 and incubated with aeration until the cell count had reached 2×10^8/ml. ($2\frac{1}{2}$–3 hours). Sufficient $CaCl_2$ was added to give a concentration of 200 μg/ml. Phage suspensions of known titre had been prepared in 5 ml. of broth. To each of these were added 10 ml. of the broth culture. A control tube without phage was included in each series and the tubes were incubated at 37° C. in the water bath. After varying times depending on the experiment but always within the latent period, the tubes were centrifuged for 2–3 minutes in an angle head centrifuge. A sample of the supernatant was taken for titration of unadsorbed phage, the supernatant then discarded and the infected cells resuspended in 2 ml. of 0·2 p.c. formol saline. This stopped the process of phage multiplication and subsequent lysis of the cells. These cell suspensions were then used for the agglutination tests.

The phage was freshly prepared 1–2 days before use to ensure that it contained little or no inactive phage that might adsorb but be incapable of penetration. Its titre of infective particles was determined the day before the experiment and again on the day of the experiment.

The number of particles adsorbed by each 10 ml. of cells was determined from the stock phage count and from the count of unadsorbed phage in the supernatant and the phage: cell ratio calculated accordingly.

The remainder of the bulk culture was infected with sufficient phage to give a one step growth curve and lysis allowed to proceed in normal fashion. This control ensured that the cells were capable of supporting phage multiplication.

The high titre neutralizing antisera previously described were used for agglutination. Before use they were adsorbed with heavy suspensions of strain 284 to remove any staphylococcal agglutinins. An anti-staphylococcal serum was included in each titration. Dilutions of serum of 1/10, 1/100, 1/1,000, 1/10,000 and 1/100,000 were made in 0·85 p.c. saline and 0·1 ml. quantities distributed to a series of agglutination tubes. Each tube in the series then received 0·1 ml. of the appropriate cell suspension. The tubes were incubated in the water bath at 37° C. for 2 hours and then left overnight on the bench before the final reading. All degrees of agglutination were noted with a hand lens.

Results.

The results of a typical experiment are given in Table 7. The phage multiplication was stopped 20 minutes after addition of the phage, 97 p.c. of the phage having been adsorbed. The antiphage serum used had an infectivity neutralizing titre of 1 in 200,000 and at a titre of 1 in 100,000 agglutinated cells to which 2 phage particles had been adsorbed. Cell suspensions with phage : cell ratios of 1:1, 1:2 and 1:4 were agglutinated by the 1 in 10,000 dilution of serum. Another serum with a neutralizing titre of 1 in 60,000 agglutinated these cells at a 1 in 10,000 dilution. Cell suspensions with a phage : cell ratio of 1:8 and the control uninfected cells were not agglutinated by the antiphage sera.

Portion at least of the infecting particle could therefore be detected on the cell surface 20 minutes after the commencement of the latent period. Since the neutralizing and agglutinating titres of the antisera corresponded closely it was concluded that the neutralizing antibody was responsible for the agglutination.

TABLE 7.

Agglutination of phage-infected cells with antiphage sera and with antistaphylococcal serum. Cell suspensions prepared in formol saline 20 minutes after infection.

Cell suspension	Antiphage agglutination					Antistaph. agglutination		Saline Control
Phage / cells Ratio	Serum dilution 1/10	1/100	1/1,000	1/10,000	1/100,000	Serum dilution 1/10	1/100	
2:1	++++	++++	+++	++	+	++++	+	—
1:1	++++	++++	+++	++	—	++++	+	—
1:2	+++	++	++	++	—	++++	+	—
1:4	+++	+	+	+	—	++++	+	—
1:8	—	—	—	—	—	+++	+	—
No phage	—	—	—	—	—	++++	+	—

Agglutination of cell suspensions with phage : cell ratios of 1:2 and 1:4 can be explained by the morphology of the cocci. The 1:2 ratio suspensions will contain large numbers of cocci in pairs which will act as single units in agglutination. The degree of visible agglutination was less in the 1:4 suspensions and the tubes from 1 in 100 onwards contained unagglutinated cells as well as agglutinated. In suspensions containing less than 1 particle to every 4 cells there will be insufficient sensitized cells to produce visible agglutination.

The presence of 2 particles per cell is insufficient to interfere with the agglutination of the cells by staphylococcal antiserum. Other experiments showed that phage : cell ratios of 3:1 or more prevented this staphylococcal agglutination, presumably by mechanical blocking of the staphylococcal surface antigen.

The stage in the latent period at which the infected cells were prepared for agglutination had no effect on their agglutinability. A bulk culture was infected at a phage : cell ratio of 1:1·2 and the cells from 10 ml. samples placed in formol saline after 15, 23, 30, 38 and 45 minutes. All samples of cells were agglutinated by a 1 in 10,000 dilution of antiserum.

It was concluded that in this particular phage-host system, the entire phage particle does not penetrate the cell after adsorption but that portion of it remains on the cell surface where it can be detected serologically throughout the latent period. Since it was the phage neutralizing antibody that detected the presence of this phage material on the cell surface, it follows that this material corresponds to the antigen that reacts in infectivity neutralizing titrations.

Intracellular Multiplication.

One of the chief objects in examining the 3A/284 system was to see whether the results obtained with coli-T5 could be repeated with a different phage-host system. The complement fixing antiserum R201 was therefore

used as a serological tool for the detection of complement fixing antigen in cells infected with 3A and lysed by sodium cyanide during the latent period. The antigen content of the lysates was titrated with a 1 in 8 dilution of this antiserum as antibody, in the presence of 2MHD of complement, the antiserum having been previously adsorbed with cells of 284 to remove staphylococcal antibodies.

Cyanide lysis of the 3A/284 system.

The technique of obtaining cyanide lysates for titration of complement fixing antigen and infective phage was similar to that used with the coli-T5 system. After the establishment of multiple infection in a bulk culture grown with aeration to $1–2 \times 10^8$ cells/ml., and to which $CaCl_2$ had been added, 10 ml. samples were withdrawn at intervals during the latent period. These were centrifuged and resuspended in 1 ml. of broth or of a glucose salt solution (Adams, 1949) to which 0·1 ml. of 0·2M NaCN was added. This concentration of cells was necessary in order to obtain antigen in sufficient quantity to be measured serologically.

The samples were cooled in an icewater bath for 30 minutes and then incubated for 45 minutes in the waterbath at 37° C. Smears were taken for Gram staining at the end of this incubation, the tubes were centrifuged and titrations of infective phage made. The complement fixing antigen titrations were made on the following day after the lysates had been stored in the refrigrator overnight.

In the first series of experiments carried out, the results indicated that 3A infected cells could be lysed by cyanide throughout the latent period. Later, however, when new batches of broth were used for growing the cells and phage, these results could not be repeated and it appeared that the cells were resistant to lysis until 18–20 minutes of the latent period had passed. The batch of broth used in the first series was the remainder of a large stock that had been prepared for the T5 experiments. T5 infected cells grown in this broth were lysable by cyanide throughout the latent period in contrast to the experience of Weigle and Delbrück (1951) who found T5 infected cells lysable only when one mature particle per cell had developed. It seems therefore that certain unknown factors in the medium may affect the lysability of the infected cells.

Although these early results could not be repeated, they were of interest since they showed the disappearance of the complement fixing antigen early in the latent period. In one experiment sufficient phage was added to the cells to give fixation at a titre of 1 in 22 but a cell sample transferred to cyanide at 12 minutes gave a titre of 1 in 4 and a sample taken 10 minutes later had a titre of only 1 in 6. No infective phage other than that which could be accounted for by unadsorbed phage could be detected in these early samples and smears from the lysates showed only Gram negative debris.

In the later experiments, cells transferred to cyanide up to 18 minutes after addition of the phage were resistant to lysis; Gram stains of the cells showed intact cocci and if the cells were plated out plaques were formed. After 20 minutes of the latent period had passed, the cyanide lysates contained a reduced number of intact cocci. At 30 minutes samples contained only Gram negative debris. Macroscopically the samples taken between 20 and 30 minutes were progressively more translucent after cyanide treatment. When they were examined after storage in the refrigerator overnight they contained only Gram negative debris indicating that prolonged contact with cyanide in the cold had disintegrated the cells not previously lysed. Lysates in which complete disintegration of the cells had occurred were translucent in appearance and slightly viscid on pipetting.

TABLE 8.

Release of complement fixing antigen and infective phage from cyanide lysed cells infected with 3A.

Time into Na/CN minutes	Cyanide lysates	
	Infective phage/10 ml.	Antigen titre/10 ml.
19	1×10^7	12
23	$1{\cdot}3 \times 10^7$	24
28	$4{\cdot}2 \times 10^8$	28
32	$6{\cdot}4 \times 10^8$	32
38	1×10^9	64
43	$1{\cdot}9 \times 10^9$	64
49	$3{\cdot}4 \times 10^9$	64
Final yield/ml.	$9{\cdot}5 \times 10^9$	7

Initial cell count, 1×10^8/ml. Multiplicity of infection, 5.
Average burst size, 95/cell.
Final yield, $9{\cdot}5 \times 10^9$/ml. Complement fixing antigen, 1 in 7.

Table 8 gives the results of a typical experiment in which the cells were grown to 1×10^8/ml. before infection and in which the initial phage : cell ratio was 5:1. Each 10 ml. sample contained 1×10^9 cells infected with 5×10^9 phage particles. Clearing of the bulk culture commenced at 60 minutes and was complete at 95 minutes. The final titre of phage was $9{\cdot}5 \times 10^9$/ml., giving an average burst size of 95.

The 19 and 23 minute samples still contained intact cocci on removal from the 37° C. water bath, but most of these had disappeared after storage overnight. All the later samples were lysed in the 37° C. water bath. Complement fixing antigen at a titre of 1 in 12 was found in the 19 minute lysate. This was higher than that found in the first series of experiments and further illustrated the variability of different batches of nutrient broth. Synthesis of the complement fixing antigen continued until 38 minutes when it reached a titre of 1 in 64. This was sufficient to account for the complement fixing anti-

gen titre of the final yield of phage. The cleared culture fixed at 1 in 7, the tenfold difference being due to the fact that the phage yield was not concentrated in the bulk culture.

The amounts of complete infective phage found in the supernatants of the 19 and 23 minute lysates were small. If the curve for infective phage given in Fig. 4 is extrapolated back to these times, the theoretical values for infective phage are much higher than those actually found. Since these 19 and 23 minute lysates contained intact cocci which were spun down before the supernatants were assayed it was probable that some of the phage filled by cyanide lysis had been readsorbed on to these cocci and so removed before titration.

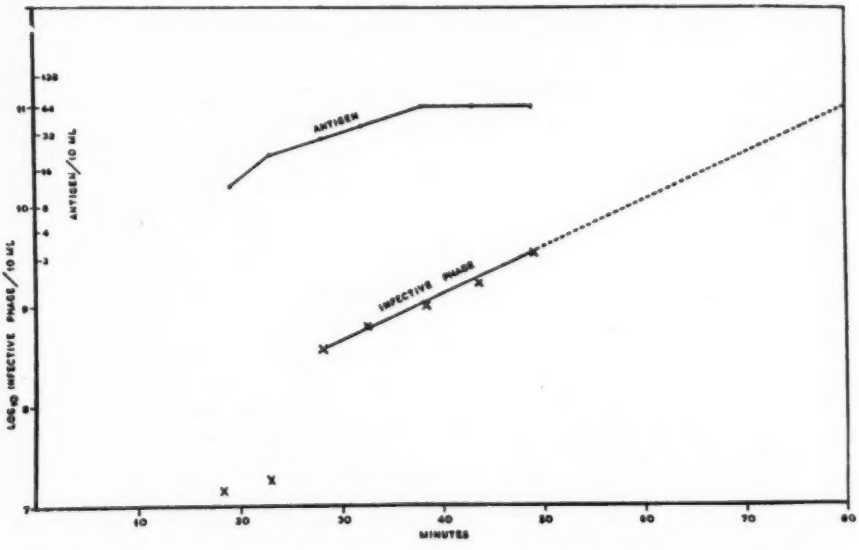

Fig. 4. Recovery of complement fixing antigen and of infective phage from cyanide lysed cells during the latent period. Initial dose of phage, 5×10^8/ml.

In the 28 minute lysate, however, complete disintegration of all the cocci occurred at 37° C. $4{\cdot}2 \times 10^8$ infective particles were obtained in this lysate indicating that only 40 p.c. of the cells each contained one infective particle at this stage of the growth period. It seems, therefore, that susceptibility to cyanide lysis in this system may not depend on the presence of a complete infective particle in every cell.

Increasing yields of infective particles were obtained as the time in the latent period increased, there being an approximate doubling of the yield at each 5 minute interval. At 49 minutes just prior to the first release of phage

in the bulk culture the average content of infective particles was 3·4/cell. If the curve given for infective phage in Fig. 4 is extrapolated it reaches the value of the final yield at 80 minutes. At 60 minutes, the point where turbidometric clearing of the culture was commencing, 10 p.c. of the final yield will have been formed. The remaining 90 p.c. will be formed between 60 and 80 minutes. There is therefore definite evidence that the rate of increase of infective phage in a bulk culture is logarithmic in character. The experiments, however, do not tell us what occurs in any individual cell in the culture.

From a number of experiments it was concluded that synthesis of the complement fixing antigen was completed 38–40 minutes after infection was initiated. From the data obtained one cannot be certain at what time it commenced. However, the early results suggested a breakdown of the complement fixing antigen early in the latent period and its reappearance in measurable amounts at 16–18 minutes. Complete infective particles appeared in substantial numbers only after the completion of this antigen synthesis.

Having regard to the longer latent period of the 3A/284 system, it seems that the course of phage multiplication as measured by C.F. antigen and infective phage synthesis is essentially similar to that found with T5.

The nature of the complement fixing antigen.

The nature of the complement fixing antigen is of considerable interest. The data already presented indicate that it is distinct from the material which reacts with the antibody in titrations designed to measure the ability to neutralize phage infectivity.

The complement fixing antigen freed by cyanide lysis at times when only few infective particles have been formed in the infected cells lacks the ability to adsorb to staphylococci. A 38 minute cyanide lysate containing 2×10^9 infective particles/ml. and having a complement fixing antigen titre of 1 in 64 was mixed with an equal volume of an 18 hour broth culture of 284 and then centrifuged. Allowing for the twofold dilution of the lysate, there was no resultant reduction in the antigen titre while the infective particles were reduced by 50 p.c.

If the complement fixing antigen is fully antigenic and if cyanide lysates containing only this antigen were obtainable, immunization of animals with such material should result in antisera containing only complement fixing antibody. Unfortunately it has not been possible to prepare such material. However, a dose of cyanide lysate containing complement fixing antigen equivalent to 7×10^{10} particles and only 3×10^8 infective particles was given to two rabbits, R422 and R423. The production of complement fixing and neutralizing antibody was followed by bleeding at appropriate intervals. Table 9 shows that these animals produced neutralizing antibody at titres of only 1 in 170 and 1 in 150 compared with the previous animals R204, R205 and R206

who received a large dose of infective particles and in whom this antibody rose to titres of 1 in 6,000. On the other hand, R422 gave a normal complement fixing antibody response, the titre reaching 1 in 190 on the 9th day and thereafter declining. In R423 the antibody response was less, reaching only 1 in 48.

TABLE 9.

Antibody response to cyanide lysate containing 3×10^8 *infective particles and complement fixing antigen equivalent to* 8×10^{10} *particles.*

	Rabbit 422 Antibody titre		Rabbit 423 Antibody titre	
Day	Neut.	C.F.	Neut.	C.F.
7	6	56	2	28
8	30	96	6	32
9	70	190	40	48
14	90	96	50	48
22	170	24	150	32

These results support the hypothesis that the complete infective particle contains more than one antigen but are not conclusive as to whether the complement fixing material can function as a complete antigen.

The chemical nature of the antigens is as yet unknown but work on this aspect of the problem is in progress.

DISCUSSION.

The previous work on the complement fixing antigen of T5 is open to the criticism that unadsorbed serum was used in titrating the antigen released from the cells by cyanide lysis. In the work now reported this source of possible error has been eliminated by the use of adsorbed sera and the results obtained indicate that in both systems the synthesis of complement fixing material takes place at a stage of phage growth earlier than the maturation of complete infective particles.

To account for the data obtained by rabbit immunization, by agglutination of infected cells and by cyanide lysis, the hypothesis is advanced that phage 3A contains two antigenic substances. A similar hypothesis could be applicable to T5.

The antigen of 3A that determines the serological group of the phage and which is not found in phages of other serological groups is measured by neutralization of infectivity of the complete phage particle. Portion, if not all of it, remains on the surface of the infected cell to which the phage has been adsorbed. It has been measured inside the infected cell only as the complete infective particle appearing in the last part of the growth cycle. The data presented do not, however, exclude the possibility that it is synthesized from

the inception of infection but has not been detected in the early stages of growth by the methods used. A satisfying morphological concept would be to identify this antigen with the envelope and the tail of the phage and it will be called for convenience the "tail" antigen without, however, implying that its correspondence with the tail has yet been demonstrated.

The antigen that is measured by complement fixation appears to be much less specific than the "tail" antigen since marked cross reactions occur between anti-3A sera and phages of other serological groups. The antigen as released from prematurely lysed cells does not adsorb to the surface of the staphylococcal cell. There is no definite evidence as to what happens to this antigen in the early stages of phage growth although there were indications from some experiments that its antigenicity disappeared during the first 20 minutes of the latent period. It is postulated that this material enters the cell and initiates the processes of phage synthesis. A convenient working hypothesis would be that it is contained in the head of the phage and is nucleic acid.

This concept of two distinct antigens in the 3A phage accords with the evidence of Herriott (1951) for the presence in T2 phage of two distinct entities that can be separated by osmotic shock. Osmotic shock produced "ghosts" consisting of the deflated head and tail of the phage. These would adsorb on to coli cells but could not cause the production of new phage in such cells. They apparently contained no nucleic acid since their chemical analysis revealed little or no phosphorus.

If the hypothesis that it is the nucleic acid portion of the phage particle that penetrates the cell is correct, then a comparison of phage growth and the type transformation of the pneumococcus and of *H. influenzae* is pertinent. In both types of phenomena, a series of processes resulting in the synthesis of a type of protein not previously present in the cell is initiated by the entrance into the cell of nucleic acid. In type transformation these syntheses lead to the production of a new kind of polysaccharide capsule and the transformed cell is not destroyed. In the growth of lytic phages, however, the cell is destroyed by the newly formed phage particles. The situation in lysogenic cells carrying phage differs from this in that the carried phage does not normally destroy the cells.

It may be suggested that the complement fixing antigen could be the form in which the phage exists in the lysogenic cell and could correspond to the pro-phage of Lwoff, Simonovitch and Kjeldgaard (1950). It would be of considerable interest to study its synthesis in lysogenic cultures in which induced lysis occurs.

SUMMARY.

The growth of a staphylococcal bacteriophage, 3A, has been examined by one step growth curves and turbidometrically in aerated broth cultures. Its minimum latent period is 50 minutes, the step being completed at 90 minutes with an average burst size of 100 phage particles.

Rabbits immunized with this phage produced two types of antibody, one neutralizing the infectivity of phage and the other giving complement fixation with phage prepared in broth. There was no correlation between neutralizing antibody titre and complement fixing antibody titre.

Phage neutralizing antisera caused specific agglutination of cells infected with an average of 0·5 particles per cell, indicating that portion of the phage remained on the surface of the infected cell after infection.

The synthesis of the complement fixing antigen and of infective particles was studied in cells lysed by cyanide during the latent period. Synthesis of complement fixing antigen was completed at 38 minutes but the data obtained were insufficient to say with certainty at what time it commenced. The number of infective particles increased in exponential fashion in the last part of the latent period.

It is postulated that the phage contains two antigens. One is associated with the ability to adsorb to cell surfaces and is measured in infectivity neutralizing titrations. The other is the complement fixing antigen and does not adsorb to cell surfaces.

The nature of the complement fixing antigen is discussed.

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THE OCCURRENCE OF AZOTOBACTER IN TWO SOUTH AUSTRALIAN SOILS

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WITH STATISTICAL APPENDIX

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The distribution of Azotobacter in Australian soils has received only sporadic attention and in consequence little is known of the numbers and activity of this organism in the majority of soils.

The first report of the isolation of an Australian strain of *Azotobacter chroococcum* was by Greig Smith (1906) who showed that the organism actively fixed nitrogen in pure culture. Darnell-Smith (1909, 1910) isolated a number of strains of Azotobacter from Victorian soils and claimed that morphological differences could be used to distinguish between organisms isolated from various soils, but he did not include descriptions of any of the organisms to substantiate this claim. Lewcock (1925), in a series of qualitative experiments, showed that Azotobacter species were widespread in South Australian soils. Beck (1935) was unable to confirm these results and reported that only 30 p.c. of the soils tested contained Azotobacter cells, the soil pH appearing to be the only limiting factor, however.

Using a quantitative agar plate count method Swaby (1939) carried out a survey of 80 Victorian soils and from his results concluded that Azotobacter was comparatively rare in most soils. Counts of the same order were obtained in a series of very thorough and careful experiments made by Jensen (1940) when he tested a number of wheat soils from N.S.W. and Victoria. Jensen found that even the positive soils contained few cells per gm.

The present study was carried out on a poor soil from the Hundred of Seddon, Kangaroo Island, and a wheat soil from the Waite Agricultural Research Institute (Urrbrae). The second soil was used in the nature of a control to determine any quantitative difference between the Azotobacter flora of the two soils, and to show any correlation between the number of nitrogen fixing bacteria in the soil and fertility. The counts of both soils were higher than

expected from previous workers' results and this increase was shown to be mostly due to the use of Martin's mannite agar for the counts.

Materials and Methods.

Fresh samples of cultivated soil were collected from the experimental farm at Parndana, Kangaroo Island (Seddon soil), packed in labelled cloth bags and sent to Adelaide by air. Samples from the 0" to 2" and 2" to 6" as well as 0" to 6" horizons were tested. The soil was a gravelly, sandy loam with a pH between 6·3 and 6·4. The Urrbrae cultivated soil (0" to 2" and 2" to 6" horizons) was collected from the wheat-fallow experimental plots at the Waite Agricultural Research Institute, Adelaide. The soil was a clay loam with a pH 5·9 to 6·0 and was also packed in cloth bags for transport to the laboratory.

The soil samples were sieved through a 2 mm. mesh to remove the stones and roots and were then stored in air-tight tins at room temperature. The soil was thoroughly mixed and a composite sample selected for testing.

Qualitative tests. Martin's liquid mannite medium was dispensed in 20 to 30 ml. amounts in 250 ml. Erlenmeyer flasks. One gm. of soil was added as the inoculum, the flask incubated at 30° C. for five to seven days and the growth examined by Gram stain.

Quantitative plate counts. The technique was a modification of that described by Swaby (1939). Martin's mannite agar containing A. R. grade reagents and washed agar was used. Calcium carbonate was not added to the agar as it made accurate colony counts impossible. Twenty gm. of soil were suspended in 200 ml. of sterile double distilled water containing 20 to 30 small round glass beads. Double dilutions in sterile distilled water were then prepared up to 1/1,280, each dilution being shaken for three minutes before preparing the next. The colonies were counted and the number of nitrogen fixing bacteria per gm. of soil calculated. From each count a number of suitable plates was selected and 25 to 30 presumed Azotobacter colonies were removed with a loop on to mannite slopes for further study.

Comparison of Martin's agar with Ashby's agar. Twenty gm. of Seddon soil were shaken for five minutes with 200 ml. of sterile double distilled water containing glass beads, and from this suspension tenfold dilutions were prepared. Ten ml. of Martin's or Ashby's agar was added to each 10 cm. Petri dish. Ten plates were used for each dilution tested. One ml. of the 1/1,000 soil dilution was added to each of the 20 plates and the inoculum solidified with two ml. of agar held at 45° C. in a water bath. The experiment was repeated using five replicate soil samples under identical conditions. After five days' incubation at 30° C. the number of Azotobacter colonies on each plate was counted.

Comparison of plate counts on soil shaken in water with and without glass beads. Urrbrae soil was diluted from 1/10 to 1/1,280 in sterile distilled water containing 15 to 20 small glass beads. At the same time a replicate sample of soil was diluted in sterile double distilled water alone. One ml. of the 1/320 and the 1/1,280 dilutions was plated in Martin's mannite agar using five plates each, and after five days' incubation at 30° C. the number of Azotobacter colonies on each plate was counted.

All plate counts were made using a Spencer dark ground plate counter.

RESULTS.

Qualitative tests for Azotobacter.

Growth of Azotobacter occurred in liquid media in five to seven days from Urrbrae or Seddon cultivated soil. The organisms formed a slimy deposit and a ring of surface growth on the walls of the flask but no surface pellicle. The

mucoid deposit was white or grey but the liquid remained clear and colourless. When the growth was plated out on Martin's mannite agar, large, round, raised, mucoid white or brown colonies developed after two days. The colonies spread on further incubation and tended to coalesce.

Smears of the growth from liquid or solid media stained by the Gram method showed large, swollen ovoid Gram negative rods and diplococci embedded in mucoid material. Slight differences in the morphology of organisms isolated from the two soils were noted.

Quantitative plate counts for Azotobacter.

Urrbrae cultivated soil (0″ to 2″ and 2″ to 6″ horizons) produced from 110,000 to 170,000 nitrogen fixing colonies per gm. of soil. The numbers were high enough to render the plates uncountable using the dilutions recommended by Swaby (1939) and soil dilutions of 1/1,000 or more were used. For the same reason the soil colony counts carried out on the 20 cm. aluminium dishes were uncountable and this technique was discarded except with two of the Seddon soil samples.

The Seddon cultivated soil (0″ to 6″ horizon) produced from 1,000 to 3,000 nitrogen fixing colonies per gm. of soil. The results of the plate counts are given in Table 1.

TABLE 1.

Numbers of aerobic nitrogen fixing bacteria in Urrbrae and Seddon soils.

Soil	Horizon	Date of sampling	Date of testing	Plate counts	
				Total number per gm.	Soil colonies per gm.
Urrbrae	0″–2″	7/3/51	8/3/51	170,000	—
Urrbrae	0″–2″	7/3/51	20/3/51	150,000	—
Urrbrae	0″–2″	11/7/51	23/7/51	170,000	—
Urrbrae	2″–6″	11/7/51	12/7/51	110,000	—
Seddon	0″–6″	24/4/51	25/4/51	2,800	—
Seddon	0″–2″	24/4/51	1/5/51	1,400	—
Seddon	0″–6″	24/4/51	10/5/51	3,400	1,000
Seddon	0″–6″	28/6/51	29/6/51	3,000	100

— = not tested.

The presumed Azotobacter colonies were distinguished from the oligonitriphiles by colonial appearance. The Azotobacter-like organisms grew in deep culture as smooth, shiny, dense colonies with a sharply defined, entire, round edge while surface colonies were larger and had a smooth, mucoid, raised surface. The colonies were translucent or white but no brown pigmented colonies were seen. The oligonitriphile colonies were flat and diffuse with an irregular, often non-entire edge. They were not as dense as the presumed Azotobacter colonies

and were usually translucent. After a little practice the two types of organisms were distinguished quite easily. Eighty p.c. of the presumed Azotobacter colonies picked off mannite agar plates for further study morphologically resembled *A. chroococcum*.

The above figures were many times greater than those obtained by previous Australian workers so the effect on the counts of the modifications introduced into the original method was tested using a sample of Urrbrae cultivated soil. A statistically significant increase in the counts carried out on Martin's agar as compared with Ashby's agar was obtained. Typical results of five replicate counts are recorded in Table 2.

TABLE 2.

Comparison of Martin's and Ashby's agars for plate counts of nitrogen fixing bacteria.

Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	
A	M	A	M	A	M	A	M	A	M
22	230	36	245	22	155	48	240	15	220
35	205	31	230	18	215	42	230	28	200
34	219	50	200	20	200	30	240	17	165
50	180	58	235	31	220	42	185	28	270
34	195	44	250	32	180	50	235	24	180
30	215	15	215	23	150	46	215	23	160
36	160	30	280	17	175	42	230	26	195
37	230	23	195	31	170	42	180	30	175
27	180	26	155	40	150	49	190	27	180
32	200	38	180	31	150	33	180	41	170

A = Number of colonies of nitrogen fixing bacteria per plate using Ashby's mannite agar.

M = Number of colonies of nitrogen fixing bacteria per plate using Martin's mannite agar.

The increase in the counts by the use of Martin's mannite agar was 530 p.c. ± 80 p.c. in terms of the number of colonies of nitrogen fixing bacteria on the Ashby's agar.

When plate counts using soil suspensions prepared in distilled water shaken with glass beads were compared with those from suspensions prepared without glass beads a statistically significant increase in the number of nitrogen fixing colonies on plates inoculated with the former material was also recorded. Typical results of five replicate counts are given in Table 3.

The increase in the counts by the use of glass beads was 44 p.c. ± 16 p.c. in terms of the number of colonies of nitrogen fixing bacteria in the "without glass beads" counts.

From the 110 presumed Azotobacter colonies picked from suitable plates and grown on nitrogen-free mannite agar until pure, 78 strains of nitrogen fixing bacteria were selected and their cultural and morphological characters determined. Thirty-four of the cultures tested morphologically and culturally

resembled *A. chroococcum,* Beijerinck, but only 15 of these produced a brown melanin pigment. The 34 organisms were presumed to be strains of *A. chroococcum,* the colourless organisms being non-pigmented variants. The remaining 44 organisms were divided into two groups which differed from *A. chroococcum* by one or more characteristics. The first group showed the typical morphology but utilized only a limited range of the 32 carbohydrates tested whilst the other group showed similar biochemical reactions to *A. chroococcum* but varied widely in morphology (Collins, 1952). The exact identity of these organisms was not determined but they grew luxuriantly on Martin's mannite agar and were presumably active nitrogen fixers.

TABLE 3.

Plate counts made on soil shaken in water with and without glass beads.

Soil sample	Treatment	Number of nitrogen fixing colonies				
		Plate 1	Plate 2	Plate 3	Plate 4	Plate 5
First replicate*	without glass beads	254	224	216	234	234
First replicate*	with glass beads	336	378	364	284	306
Second replicate*	without glass beads	346	212	184	222	236
Second replicate*	with glass beads	256	248	412	364	376
Third replicate*	without glass beads	288	250	272	232	238
Third replicate*	with glass beads	398	334	332	392	374
Fourth replicate*	without glass beads	210	258	224	212	214
Fourth replicate*	with glass beads	316	366	422	372	250
Fifth replicate*	without glass beads	254	244	266	224	226
Fifth replicate*	with glass beads	272	372	358	292	344

*All plates were inoculated with 1 ml. of a $\frac{1}{640}$ dilution of soil.

DISCUSSION.

The present study was undertaken to determine any connection between the number of aerobic nitrogen fixing bacteria in the Seddon and Urrbrae virgin soils and the difference in their fertility. The low fertility of the Seddon soil despite a good rainfall was thought partly to be due to its low powers of nitrogen fixation.

The first step in the investigation was the qualitative demonstration of Azotobacter in both soils. Azotobacter-like organisms were isolated from both soils without difficulty and the majority closely resembled *A. chroococcum.* Only 10 p.c. of the strains produced a brown melanin pigment, the others forming white or translucent colonies. A few of the strains failed completely to grow in liquid media but the majority formed a surface ring of growth on the walls of the flask or tube. Lewcock (1925) reported the isolation from a South Australian soil of a strain of Azotobacter unable to grow in liquid media and this type of organism may be relatively common in these soils.

One striking characteristic of *A. chroococcum*, Beijerinck, was the ability to form a thin, tenaceous, brown surface pellicle in liquid media or large, moist, brown colonies on mannite agar. The absence of pigment from the great majority of Azotobacter-like organisms caused some doubt regarding their identity but, since they morphologically and culturally resembled *A. chroococcum*, they were classed as non-pigmented strains of this organism. Starkey (1951) stated that a number of non-pigmented strains of *A. chroococcum* had been isolated from soil and that non-pigmented strains could be obtained from previously pigmented organisms. Therefore, less importance was attached to pigment production than to biochemical and morphological criteria in typing the organisms.

The hundredfold difference between the number of nitrogen fixing bacteria in the two soils may have had some effect on the amount of nitrogen fixed in the soil, although Jensen (1940) failed to detect increases in soil nitrogen even after multiplication of Azotobacter had been shown by viable counts. There did not seem to be any obvious reason for the large difference between the counts and since the pH of both soils was in the region of 6·0 this factor was unlikely to be limiting.

Reports by previous Australian workers tended to indicate that Azotobacter was rarely present in most soils. Beck (1935) reported an average of 86 Azotobacter per gm. with a maximum of 560 per gm. in a Findon soil. Swaby (1939) obtained counts of 30 or more Azotobacter per gm. in only 10 p.c. of the samples, with a maximum count of 1,500 cells per gm., while Jensen (1940) found up to 2,300 Azotobacter per gm. in one sample but most of the soils tested contained few, if any, Azotobacter.

The high counts obtained in the present study were unexpected and steps were taken to make sure that oligonitriphile colonies were not being mistaken for Azotobacter colonies. The oligonitriphiles grew on small amounts of fixed nitrogen in the agar but on plating them on Martin's agar growth was scanty and disappeared altogether after two subcultures. The presumed Azotobacter formed luxuriant growth on Martin's agar through numerous subcultures. Attempts were made to decrease the amount of available nitrogen in the agar and in this way prevent the growth of all except the bacteria actively fixing atmospheric nitrogen. Following a suggestion by Swaby (1951) washed agar and A.R. reagents were used to prepare the Martin's mannite agar. Martin (1940) reported the use of commercial cane sugar to supply trace elements and growth factors as well as an additional energy supply to the medium and so improved the growth of Azotobacter. When this medium was prepared during the present work it was shown to contain appreciable amounts of nitrogen, but when A.R. grade sucrose was used in place of cane sugar the agar was no longer able to support the growth of non-nitrogen fixing bacteria. The sucrose probably assisted the growth of nitrogen fixing bacteria which were unable to utilize

mannite and together with the molybdenum in the medium may have been responsible for most of the 500 p.c. increase in plate counts. van Niel (1935) reported that the addition of molybdenum to nitrogen-free media showed the presence of Azotobacter in soils from which they had previously been thought to be absent. Jensen (1940, 1947) used molybdenum in his medium for plate counts and he later showed that molybdenum was essential for nitrogen fixation as well as growth of Azotobacter. Since Jensen's counts were made using media containing molybdenum it was apparently the sucrose in Martin's agar that was responsible for the majority of the 500 p.c. increase although the reason for this stimulation was not obvious.

The 500 p.c. increase by the use of Martin's agar combined with the 44 p.c. increase when the soil suspension was shaken with glass beads accounted for most of the difference between the present results and those of Swaby and of Jensen. Calculation showed that use of Swaby's unmodified technique would have probably given counts of 20,000 to 30,000 aerobic nitrogen fixing bacteria per gm. of Urrbrae cultivated soil and 400 to 1,200 nitrogen fixers per gm. of Seddon cultivated soil. The higher values of the present counts were not entirely due to the changes in technique since the two increases were unable to account for all the difference which may have been partly due to other variations not studied or to the use of different soil samples.

Approximately 50 p.c. of the presumed Azotobacter strains studied in pure culture resembled *A. chroococcum* but no definite conclusion was reached regarding the identity of the remainder. The identification of these organisms, while of considerable academic interest, seemed less important than the fact that they were presumably active nitrogen fixers, being able to grow indefinitely on nitrogen-free media. The higher counts obtained in the present work may be of some practical importance since Jensen (1940) concluded from his survey of N.S.W. wheat soils that acid soils contained only small numbers of organisms and had slight nitrogen fixing powers, so that at the most only one-third of the crop nitrogen requirements could be supplied by the nitrogen fixers under the most favourable conditions. The larger flora of these two South Australian soils may indicate an explanation of the continued fertility of wheat soils even after long and continuous cropping without the addition of nitrogenous fertilizers. Even if the nitrogen fixers were unable to replace all the nitrogen removed from the soil during cropping they may replace sufficient to make depletion of available nitrogen a very long process.

SUMMARY.

Plate counts of aerobic nitrogen fixing bacteria in Urrbrae and Seddon cultivated soils were higher than those reported by previous Australian workers.

Over 500 p.c. increase in plate counts following the use of Martin's mannite agar instead of Ashby's agar and 44 p.c. increase following the use of glass beads during the preparation of the inoculum accounted for most of the difference.

Pure culture studies of 110 presumed Azotobacter organisms showed that the majority resembled *A. chroococcum* either culturally or morphologically, or both.

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APPENDIX.

Comparison of the two agars.

On the assumption that the counts are distributed according to the Poisson distribution the square-root transformation was applied to these data. It proved successful in stabilizing the subclass variances.

The results of an analysis of variance applied on the transformed data are as follows:

Deviation due to	D.F.	S. Sq.	M. Sq.	Ratio
Agars	1	1,784·015	1,784	2,160***
Samples	4	25·611	6·403	7·757***
Interaction	4	2·694	0·674	n.s.
Residual	90	74·287	0·8254	
Total:	99	1,886·606		

The counts for the two agars differ enormously. The samples are significantly different at the 0·1 p.c. level showing that the sampling of the soil was not consistent. The absence of interaction, however, shows no differential response to agars over the five samples.

The 5 p.c. fiducial limits for the transformed means are: Ashby's 5·647 ± 0·256, Martin's 14·095 ± 0·256. Transforming back to the original variates, the limits for the means are 32·0 ± 2·9 and 119 ± 7 so that the percentage increase estimated to be 510 p.c. lies in the range 450 to 610 with a fiducial probability of approximately, and at least, 95 p.c.

Effect of beads.

The counts were again transformed in the same way and for the same reason and again stabilized the subclass variances. The resulting analysis of variance on the transformed variates is:

Deviation due to	D.F.	S. Sq.	M. Sq.	Ratio
Beads	1	119·753	119·8	86·7
Samples	4	4·637	1·159	n.s.
Interaction	4	4·081	1·020	n.s.
Residual	40	55·242	1·381	
Total:	49	183·713		

thus showing a highly significant increase in count by the use of beads.

The estimated percentage increase is 44 p.c. and in exactly the same way as before the approximate, and at least, 95 p.c. fiducial range for the percentage increase in the count is 32 p.c. to 66 p.c.